METHODS IN MOLECULAR BIOLOGY™ 354

Plant–Pathogen Interactions

Methods and Protocols

Edited by

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HUMANA PRESS

Plant–Pathogen Interactions

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Plant–Pathogen Interactions

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Edited by

Pamela C. Ronald

Department of Plant Pathology
University of California at Davis
Davis, CA

HUMANA PRESS   TOTOWA, NEW JERSEY

© 2007 Humana Press Inc.
999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

humanapress.com

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This publication is printed on acid-free paper. ∞

ANSI Z39.48-1984 (American Standards Institute)

Permanence of Paper for Printed Library Materials.
Cover illustration: Fig. 1, Chapter 1, "The Use of Protoplasts to Study Innate Immune Responses," by Ping He, Libo Shan, and Jen Sheen.

Production Editor: Amy Thau

Cover design by Patricia F. Cleary

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

eISBN: 1-59259-966-4

ISSN: 1064-3745

Library of Congress Cataloging-in-Publication Data

Plant-pathogen interactions : methods and protocols / edited by
Pamela C. Ronald.
p. ; cm. -- (Methods in molecular biology, ISSN 1064-3745 ; 354)
Includes bibliographical references and index.
ISBN-13: 978-1-58829-448-7
ISBN-10: 1-58829-448-X (alk. paper)
1. Plant-pathogen relationships--Molecular
aspects--Research--Methodology. 2. Plant diseases--Molecular
aspects--Research--Methodology.
[DNLM: 1. Plant Diseases--microbiology. 2. Gene Expression
Regulation, Plant--immunology. 3. Gene Silencing. 4. Immunity,
Natural--genetics. 5. Plant Diseases--genetics. QW 60 P7135 2006]
I. Ronald, Pamela C. II. Series: Methods in molecular biology
(Clifton, N.J.) ; v. 354.

SB732.7.P53 2006

632'.3--dc22

2006007133

Preface

More than 50 years ago, Flor *(1)* proposed a model to describe plant–pathogen interactions based on genetic studies with flax and the flax-rust pathogen. His "gene-for-gene" model predicted that plant resistance would occur only when a plant possesses a dominant resistance gene (R) and the pathogen expresses the complementary dominant avirulence gene (Avr), conferring strain specificity. An alteration or loss of the plant resistance gene or the pathogen Avr determinant leads to disease in the host. The R gene products are hypothesized to act as receptors for the products of the Avr locus. As a result of intense research in the last 10 years, it is now well established that Flor's model still holds true for many host–pathogen interactions.

We now know that components of innate immune systems in both plants and animals share many conserved features *(2)*. Most notably, they sense the presence of pathogen-associated molecular patterns, which represent conserved molecular structures, and Avr factors. Many plant bacterial pathogens use type III secretion systems to secrete proteins into host cells, where they can affect host cell metabolism and, in some cases, be detected by intracellular R proteins. In contrast, little is known about the identity, production, and secretion of pathogen-associated molecules detected at the cell surface. The first three chapters in *Plant–Pathogen Interactions: Methods and Protocols* describe methodologies being used to identify and characterize such pathogen-associated molecular patterns or Avr factors from bacteria, and the plant responses they trigger. Chapters 4 and 6 describe methods for identifying and characterizing such molecules from oomycete and fungal pathogens.

Identification of many of the first R genes was carried out by positional cloning approaches, which establish linkage of plant resistance to markers whose physical location in the genome is known. Over the last few years, major advances in plant genomics have made positional cloning in rice and *Arabidopsis* much more efficient. These methods and resources are described in Chapters 5 and 7.

Advances in genomics and proteomics led to new methods to identify genes and proteins that are potentially involved in resistance-signaling pathways. Microarrays, which consist of dense arrays of oligonucleotides attached to a solid surface such as glass, are increasingly being used as more complete arrays are produced and analytical tools are becoming easier to use. For deep transcriptome analysis, robust long-serial analysis of gene expression and massively parallel signature sequencing are the methods of choice. Of the proteomic methods, the

yeast two-hybrid system employs yeast to identify proteins that interact with a particular "bait" or plant-signaling protein. Another method applies proteomic techniques to investigate posttranscriptional changes by enriching for specific proteins before two-dimensional gel separations. These approaches are described in Chapters 8–13.

Viral-induced gene silencing and RNAi silencing can be used to quickly assess the function of a particular protein in plant leaves or plant roots. These strategies and their molecular mechanisms are described in Chapters 14–16.

The review Chapters 17 and 18 describe methods for engineering resistance to plant viruses and demonstrate the utility of this approach for development of virus-resistant crop plants of value for agriculture.

In summary, *Plant–Pathogen Interactions: Methods and Protocols* gathers together some of the key methods used in studies of plant–pathogen interactions and includes chapters describing how this knowledge is being used to develop new strategies for disease control. We hope you find it useful.

Pamela C. Ronald

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Contributors

MAWSHENG CHERN • *Department of Plant Pathology, University of California at Davis, Davis, CA*

XIAODONG DING • *Department of Plant Pathology, University of Florida, Gainesville, FL*

XIN SHUN DING • *Division of Plant Biology, Samuel Roberts Noble Foundation, Ardmore, OK*

BRYCE W. FALK • *Department of Plant Pathology, University of California at Davis, Davis, CA*

JANE GLAZEBROOK • *Department of Plant Biology, University of Minnesota, St. Paul, MN*

DENNIS GONSALVES • *US Pacific Basin Agricultural Research Center, US Department of Agriculture, Hilo, HI*

MALALI GOWDA • *Department of Plant Pathology, The Ohio State University, Columbus, OH*

JEAN T. GREENBERG • *Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL*

CHRISTIAN D. HAUDENSCHILD • *Solexa Inc., Hayward, CA*

PING HE • *Department of Molecular Biology, Massachusetts General Hospital; and Department of Genetics, Harvard Medical School, Boston, MA*

EDGAR HUITEMA • *Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH*

JONG-SEONG JEON • *Graduate School of Biotechnology and Plant Metabolism Research Center, Kyung Hee University, Yongin, Korea*

YULIN JIA • *Genomics Core Facility, Dale Bumpers National Rice Research Center, US Department of Agriculture, Agricultural Research Service, Stuttgart, AZ*

ISGOUHI KALOSHIAN • *Department of Nematology, University of California at Riverside, Riverside, CA*

THIRUMALA-DEVI KANNEGANTI • *Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH*

SOPHIEN KAMOUN • *Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH*

SANG-WON LEE • *Department of Plant Pathology, University of California at Davis, Davis, CA*

XIN LI • *Michael Smith Laboratories, Department of Botany, University of British Columbia, Vancouver, Canada*
CHUANSHENG MEI • *Department of Plant Pathology, University of Arkansas, Fayetteville, AR*
BLAKE C. MEYERS • *Department of Plant and Soil Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, DE*
RICHARD S. NELSON • *Division of Plant Biology, Samuel Roberts Noble Foundation, Ardmore, OK*
MARC J. ORBACH • *Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, AZ*
VISHAL PAMPANWAR • *Arizona Genomics Computational Laboratory, BIO5 Institute, University of Arizona, Tucson, AZ*
SCOTT C. PECK • *The Sainsbury Laboratory, John Innes Center, Norwich, United Kingdom*
GREG RAIRDAN • *Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY; and The Sainsbury Laboratory, John Innes Center, Norwich, United Kingdom*
C. SRINIVASA RAO • *Division of Plant Biology, Samuel Roberts Noble Foundation, Ardmore, OK*
TODD RICHTER • *Department of Plant Pathology, University of California at Davis, Davis, CA*
PAMELA C. RONALD • *Department of Plant Pathology, University of California at Davis, Davis, CA*
GOURGOPAL ROY • *Department of Entomology, University of California at Davis, Davis, CA*
ANTONIO SERNA-SANZ • *The Sainsbury Laboratory, John Innes Center, Norwich, United Kingdom*
LIBO SHAN • *Department of Molecular Biology, Massachusetts General Hospital; and Department of Genetics, Harvard Medical School, Boston, MA*
JEN SHEEN • *Department of Molecular Biology, Massachusetts General Hospital; and Department of Genetics, Harvard Medical School, Boston, MA*
CAROL SODERLUND • *Arizona Genomics Computational Laboratory, BIO5 Institute, University of Arizona, Tucson, AZ*
WEN-YUAN SONG • *Department of Plant Pathology, University of Florida, Gainesville, FL*
ERIC STAHLBERG • *Ohio Supercomputer Center, The Ohio State University, Columbus, OH*
MYSORE R. SUDARSHANA • *Western Institute for Food Safety and Security, University of California at Davis, Davis, CA*

Jon Suzuki • *US Pacific Basin Agricultural Research Center, US Department of Agriculture, Hilo, HI*

Savarni Tripathi • *US Pacific Basin Agricultural Research Center, US Department of Agriculture, Hilo, HI*

Sara L. Tucker • *Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, AZ*

Kalyan Vemaraju • *Department of Plant and Soil Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, DE*

R. C. Venu • *Department of Plant Pathology, The Ohio State University, Columbus, OH*

Boris A. Vinatzer • *Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA*

Guo-Liang Wang • *Department of Plant Pathology, The Ohio State University, Columbus, OH*

Yinong Yang • *Department of Plant Pathology, University of Arkansas, Fayetteville, AR*

Yan Zhang • *University of Florida, Gainesville, FL*

Yuelin Zhang • *National Institute of Biological Sciences (NIBS), Beijing, China*

Xiangjun Zhou • *Department of Plant Pathology, University of Arkansas, Fayetteville, AR*

1

The Use of Protoplasts to Study Innate Immune Responses

Ping He, Libo Shan, and Jen Sheen

Summary

The use of plant protoplast transient expression system has facilitated the discovery and dissection of many signal transduction pathways in response to hormones, metabolites, and stresses. Recently, *Arabidopsis* protoplasts also have been used successfully to study plant innate immune responses triggered by pathogen-derived elicitors. Here, we describe the detailed protocols for studying innate immune responses, including cell death and early defense gene regulation activated by two types of elicitors, pathogen-associated molecular patterns and bacterial type III effectors in *Arabidopsis* protoplasts. This cell-based system simplifies the complex pathogen–plant interactions to pure individual signals and synchronized cell-autonomous responses. The application of this novel approach provides high temporal and spatial resolution to enhance our understanding of the distinct and overlapping signaling events in pathogen-associated molecular pattern- and bacterial type III effector-activated immune responses at the molecular and cellular level.

Key Words: *Arabidopsis*; mesophyll protoplast; innate immunity; PAMP; Avr; cell death; early defense gene regulation.

1. Introduction

Plants rely on innate immune responses to launch inducible defense against bacterial, fungal, and viral pathogens upon recognition of diverse pathogen-derived elicitors. The elicitors are either conserved among several microbial species (pathogen-associated molecular patterns [PAMPs]) or specific to some races of a pathogen species (avirulence [Avr] or type III effectors). The recognition of PAMPs is likely mediated by receptor-like kinases with extracellular leucine-rich repeats. In *Arabidopsis*, *FLS2* encodes an leucine-rich-repeat-receptor-like kinase as the receptor for bacterial flagellin ***(1)***. Avr or type III effectors are recognized by plant resistance (R) proteins to trigger gene-for-

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

gene resistance ***(2)***. R proteins are associated with plasma membrane or localized in the intracellular cytosol or nucleus to directly or indirectly interact with *avr* gene products that are secreted and translocated by bacterial type III secretion system into plant cells ***(3,4)***. So far, more than 40 *R* genes have been identified in diverse plant species, but the signal transduction pathways activated by R proteins are still poorly understood ***(3,5,6)***. Extensive genetic screens have led to the isolation of many important components in gene-for-gene resistance and PAMP-mediated basal resistance ***(5–7)***. However, their biochemical functions and molecular actions in defense responses are largely unknown.

It has been widely assumed that PAMP and Avr trigger mostly convergent innate immune responses, including calcium influx, kinase activation, oxidative signaling, transcription reprogramming and, in some cases, programmed cell death ***(8,9)***. Recently, analyses of global gene expression profiles have suggested that similar defense gene expression programs are shared by compatible (disease caused by virulent bacteria) and incompatible (resistance to avirulent bacteria) plant-pathogen interactions at the genome level ***(10)***. However, because the whole plant–pathogen interactions display complex responses stimulated simultaneously by a large array of extracellular and intracellular pathogen elicitors, the traditional approach provided limited resolution in dissecting the molecular mechanisms of early defense signaling events at the cellular level.

The use of transient gene expression in a cell-based system has facilitated the rapid discoveries of signal transduction pathways in many multicellular organisms. The freshly isolated *Arabidopsis* mesophyll protoplasts display physiological and cell-autonomous responses to a broad spectrum of signals, including light, sugar, auxin, cytokinin, abscisic acid, hydrogen peroxide, and stresses, similar to those found in intact tissues and plants ***(11,12)***. These protoplasts also have been used to investigate cell death induced by a fungal elicitor fumonisin B1 and a type III effector AvrRpt2 ***(13,14)***. Notably, *Arabidopsis* mesophyll protoplasts have been developed to study plant innate immune responses, including activation of mitogen-activated protein kinase cascades and WRKY transcription factors triggered by flagellin ***(15)***. Future applications of the protoplast transient expression system could facilitate the dissection and comparison of different types of immune responses triggered by individual pathogen-derived elicitors at the cellular and molecular level. The protoplast system provides unique opportunities to explore the elusive early signaling events in plant disease resistance.

We have demonstrated that protoplasts could be transfected with bacterial *avr* genes under the control of a constitutive or inducible promoter, or treated with different PAMPs to study cell death, defense gene regulation, protein degradation and interaction, and kinase activation (**Fig. 1**). The same approach

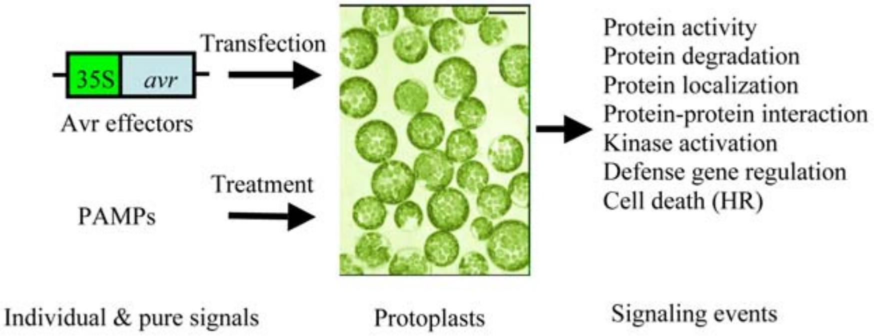

Fig. 1. The use of protoplast transient assays to study early signaling events mediated by Avr and pathogen-associated molecular pattern. 35S is the constitutive promoter derived from cauliflower mosaic virus. HR, hypersensitive response.

could be used to study the functions of R proteins and other signaling components in the defense network. In combination with genetic, genomic, proteomic, and computational tools, this powerful cell-based system will broaden our understanding the signal transduction mechanisms of plant innate immunity.

2. Materials

2.1. Construction of the Plant Expression Plasmids

1. Effector constructs: clone the desired coding region of *avr* genes, *R* genes, or other signaling genes into a plant expression vector behind the constitutive 35S promoter or an inducible promoter ***(16,17)***.
2. Reporter constructs: fuse the promoter of various target genes with a reporter gene, such as the *LUC* (firefly luciferase), *GFP* (green fluorescent protein), or *GUS* (β-glucuronidase) genes ***(16–18)***.

2.2. Protoplast Isolation and Transfection

1. Plant material: 4-wk-old *Arabidopsis* plants (Col-0 or Ler) grown in soil in the greenhouse or growth chamber (30–65% relative humidity, 20–25°C, 50–100 μ mol/m^{-2}/s light, 10- to 13-h photoperiod).
2. Enzyme solution: 1.5% cellulase R10, 0.4% macerozyme R10, 0.4 *M* mannitol, 20 m*M* KCl, 20 m*M* MES, pH 5.7
3. 0.45-μm Filter.
4. Razor blades.
5. Desiccator.
6. 35- to 75-μm nylon mesh.
7. 30-mL Round-bottom polypropylene tubes.
8. Hemacytometer.
9. 2-mL Round-bottom tubes.

10. 40% (w/v) polyethylene glycol (PEG) solution: To make 10 mL of PEG solution, add 4 g of PEG4000 (Fluka, cat. no. 81240) into 3 mL of H_2O, 2.5 mL of 0.8 *M* mannitol, and 1 mL of 1 *M* $CaCl_2$.
11. W5 solution: 154 m*M* NaCl, 125 m*M* $CaCl_2$, 5 m*M* KCl, 2 m*M* MES pH 5.7.
12. MMg solution: 0.4 *M* mannitol, 15 m*M* $MgCl_2$, 4 m*M* MES pH 5.7.
13. WI solution: 0.5 *M* mannitol, 20 m*M* KCl, 4 m*M* MES pH 5.7.
14. Tissue culture plates (6-well, 12-well, or 24-well).
15. PAMP: Flg22, the conserved 22 amino acids of flagellin, chemically synthesized according to the published peptide sequence ***(19)***.

2.3. Immune Response Assays

1. Light microscope.
2. Evans blue (Sigma).
3. Fluorescent microscope.
4. YO-PRO-1 (Molecular Probes, Y-3603).
5. 4-Methylumbelliferyl-β-D-glucuronide (MUG).
6. Fluorometer.
7. Cell lysis buffer: 25 m*M* Tris-phosphate pH 7.8, 2 m*M* 1, 2-diaminocyclohexane-*N*,*N*,*N*,*N*-tetraacetic acid, 10% glycerol, 1% Triton X-100, 2 m*M* dithiothreitol (DTT).
8. Luciferase assay substrate (Promega, E1501).
9. Luminometer (Monolight™ 3010, BD Bioscience).
10. TRIzol Reagent (Invitrogen).
11. Oligo(dT) (500 ng/μL; Invitrogen).
12. dNTP (Mix of dATP, dTTP, dGTP, and dCTP; New England Biolabs).
13. 5X first strand buffer (Invitrogen).
14. 0.1 *M* DTT (Invitrogen).
15. RNase inhibitor (40 U/μL, Invitrogen).
16. M-MLV reverse transcriptase (200 U/μL, Invitrogen).
17. RNase-free DNase I (Invitrogen).

3. Methods

3.1. Protoplast Isolation

1. Prepare enzyme solution (*see* **Note 1**).
2. Heat the enzyme solution at 55°C for 10 min to inactivate proteases and enhance enzyme solubility.
3. Cool the solution to room temperature before adding 10 m*M* $CaCl_2$ and 0.1% bovine serum albumin (Sigma, A7906).
4. Pass the solution through a 0.45-μm filter into a Petri dish.
5. Cut well-expanded *Arabidopsis* leaves (usually the middle section of the third or fourth pair of true leaves approx 1–1.5 cm in length) into 0.5-mm strips with fresh razor blades and digest the leaf strips in the enzyme solution in a Petri dish (*see* **Note 2**).
6. Cover the Petri dish with the foil and apply vacuum infiltration by using a desiccator for 30 min.

7. Continue the digestion without vacuum or shaking for another 2.5–3 h. The digestion time may vary depending on the material and experimental goals.
8. Release the protoplasts by gently shaking the Petri dish by hand or use a shaker at 80 rpm for 1 min. Be gentle with the protoplasts. Some leaves now turn transparent and the enzyme solution becomes green.
9. Add equal volume of W5 solution to facilitate protoplast centrifugation.
10. Filter the enzyme solution containing protoplasts with a 35- to 75-μm nylon mesh into a 30 mL round-bottom tube.
11. Pellet the protoplasts by spinning for 2 min at 100*g* or speed 3 using an IEC clinical centrifuge.
12. Resuspend the protoplasts in 0.5 mL of W5 solution by gently shaking.
13. Count protoplasts using a hemacytometer under the light microscope and adjust the protoplasts in the W5 solution to a density of 2×10^5 /mL.
14. Keep the protoplasts on ice for at least 30 min in the W5 solution to allow recovery from isolation stress.
15. The protoplasts should settle to the bottom of the tube in 5–10 min. Before PEG-Ca^{2+} transfection, pipet the W5 solution out and resuspend the protoplasts in MMg solution at a density of 2×10^5/mL.

3.2. PEG Transfection, PAMP Treatment, and Incubation

1. Prepare 40% (w/v) PEG solution with 0.2 *M* mannitol and 100 m*M* $CaCl_2$.
2. Take out the plasmid DNA from the –20°C freezer and thaw it completely (*see* **Note 3**).
3. Add 20 μL (20–40 μg) of the mixed effector and reporter DNA into a round-bottom 2 mL tube (*see* **Note 4**).
4. Add 200 μL of protoplasts in MMg solution prepared from **Subheading 3.1.15.** into the tube (*see* **Note 5**).
5. After adding protoplasts, immediately add 220 μL of 40% PEG into the tube and mix well gently.
6. Incubate at room temperature (23°C) for 5–30 min.
7. Stop the transfection by adding 0.8 mL W5 solution and mix well.
8. Spin at 100*g* for 2 min and remove PEG.
9. Resuspend the protoplasts gently with 100 μL WI.
10. Add the protoplasts into a six-well tissue culture plate with 1 mL of WI (*see* **Note 6**).
11. Treat the protoplasts with PAMPs (optional; *see* **Note 7**).
11. Incubate the protoplasts under desirable conditions (*see* **Note 8**).
12. After incubation for 2 to 16 h, protoplasts could be investigated immediately for cell death (*see* **Subheading 3.3.1.**), GFP expression, protein localization, or gene expression (*see* **Note 9**).
13. Alternatively, harvest protoplasts by centrifugation at 100*g* for 2 min and remove the supernatant. Freeze and store the samples at –80°C until ready for diverse assays.

3.3. Immune Response Assays

3.3.1. Cell Death Assays

3.3.1.1. Evans Blue Staining

1. Add Evans blue dye to the protoplasts in WI solution to a final concentration of 0.04%.
2. Incubate for 10 min at room temperature.
3. Determine the dead (stained blue) and viable (unstained) cells under a light microscope.

3.3.1.2. YO-PRO-1 Staining

1. Add YO-PRO-1 to the protoplasts in WI solution to a final concentration of 0.5 μ*M*.
2. Determine the dead cells (intense green fluorescence and nuclear fragmentation in the nuclei) under a fluorescent microscope.

3.3.2. Reporter Gene Assays

3.3.2.1. Luciferase Activity Assay

1. Take out the samples from –80°C freezer and add 100 μL of cell lysis buffer when they are still frozen (*see* **Note 10)**.
2. Vortex vigorously for 2 s to lyse the protoplasts and keep the lysate on ice.
3. Spin down cell debris at 8000 to 10,000*g* for 1 min at 4°C.
4. Use 5 to 50 μL of cell extract to measure luciferase activity by using luciferase assay substrate with a luminometer (*see* **Note 11)**.

3.3.2.2. GUS Activity Assay

1. Add 10 μL of cell extract prepared from **Subheading 3.3.2.1.**, **step 3**, into 90 μL of 1 m*M* MUG in 10 m*M* Tris-HCl, pH 8.0, and 2 m*M* $MgCl_2$, and mix well.
2. Incubate at 37°C for 30 to 90 min.
3. Add 0.9 mL of 0.2 *M* Na_2CO_3 to stop the reaction.
4. Measure the fluorescence of MU using a fluorometer.

3.3.3. Reverse Transcription Polymerase Chain Reaction Assay

1. Isolate total RNA by using TRIzol Reagent (Invitrogen) according to the handbook. Add 0.4 mL of TRIzol for 8×10^4 protoplasts (*see* **Note 12**).
2. Mix 1 μg of total RNA, 0.1 μL of oligo(dT) (500 ng/μL) and RNase-free H_2O in a final volume of 14 μL.
3. Heat the mix at 65 to 70°C for 5 min and chill on ice.
4. Briefly spin down the samples.
5. Add 6 μL of cDNA synthesis cocktail (4 μL of 5X first-strand buffer, 1 μL of 2.5 m*M* dNTP, 0.4 μL of 0.1 *M* DTT, 0.4 μL of RNase inhibitor, and 0.2 μL of reverse transcriptase).

6. Incubate at 42°C for 1 h.
7. Add 20 μL of H_2O.
8. Take 1 μL of the first-strand cDNA template for each polymerase chain reaction (PCR) using primers of the interested genes or control genes, such as genes encoding actin, ubiquitin, or tubulin (*see* **Notes 13–15**).
9. Alternatively, take 0.1 to 0.2 μL of complementary DNA template for real-time PCR analysis.

4. Notes

1. Prepare 10 mL of solution to digest 10 to 20 leaves, which could yield approximately one million protoplasts.
2. The growth condition of plants is most critical for experimental reproducibility. Researchers in each laboratory may need to work out the best plant growth conditions. The well-expanded third and fourth pairs of leaves are recommended for the protoplast isolation.
3. The quality of DNA is very important for protoplast transfection. Poor-quality DNA may kill protoplasts and fail to produce any results. It is recommended to use CsCl gradients for Maxi-plasmid DNA isolation. The protocol could be downloaded at http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html.
4. The ratio of effector and reporter DNA could vary from 2:1 to 4:1.
5. The experiments can be easily scaled up or down as long as the recommended DNA/protoplasts ratio is followed. Use 200 μL of (4×10^4) cells for most experiments, such as Western blot analysis and kinase activation. However, reporter enzyme assays only require 50 μL (1×10^4) cells.
6. To prevent sticking of protoplasts to the plastic, the plates could be coated with 5% calf serum for 1 second before use. You can also use 12- or 24-well tissue culture plates for small amount of cells.
7. The protoplasts could be treated with different PAMPs, such as bacterial flagellin and lipopolysaccharide, and fungal chitin.
8. The incubation conditions, such as light and temperature, depend on the purpose of experiments. For most experiments, protoplasts could be incubated at room temperature under low light (30–50 μ mol/m^{-2}/s).
9. The incubation time varies in different assays. The incubation time is 3 to 6 h for Western blot analysis and reporter enzyme assay and 1 to 6 h for reverse transcription (RT)-PCR analysis. The kinase activation could be detected within minutes after PAMP treatment.
10. Add DTT in cell lysis buffer right before use.
11. Dilute the cell extract with cell lysis buffer if the reading is over the linear range of the luminometer.
12. The RNA yield is 2 to 3 μg for 8×10^4 protoplasts, which is sufficient to analyze 40 to 50 genes by RT-PCR.
13. The number of PCR cycles depends on the abundance of the tested genes. It is usually 25 to 35 cycles.

14. It is necessary to carry out a control PCR using RNA as template without RT. If a PCR product is amplified from the control reaction, this means that there is genomic DNA contamination in your RNA samples. RNA samples could be treated with RNase-free DNase I (Invitrogen) to remove DNA before RT.
15. Try to design RT-PCR primers to cover an intron so that the size of PCR product from cDNA is smaller than that from genomic DNA, or to design one primer covering the sequences from two exons, so that the primer can only anneal to the cDNA but not genomic DNA.

Acknowledgments

This work was supported by the National Science Foundation and the National Institute of Health.

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2

Marker-Exchange Mutagenesis and Complementation Strategies for the Gram-Negative Bacteria *Xanthomonas oryzae* pv. *oryzae*

Sang-Won Lee and Pamela C. Ronald

Summary

This chapter describes methods for targeted knockouts using marker exchange mutagenesis and complementation of the Gram-negative bacteria *Xanthomonas oryzae* pv. *oryzae*. We have used these methods to demonstrate that type I secretion and modification systems are involved in avrXa21 activity of *X. oryzae* pv. *oryzae*.

Key Words: Marker-exchange mutagenesis; overexpression; Gram-negative bacteria; *Xanthomonas oryzae* pv. *oryzae*.

1. Introduction

Innate immunity provides a first line of defense against pathogen attack and is activated rapidly after infection. In contrast to the adaptive immune system that depends on somatic gene rearrangements for the generation of antigen receptors with random specificities, the innate immune system uses a set of defined receptors for pathogen recognition ***(1)***. Although it is now widely appreciated that pathogen recognition receptors play a key role in innate immunity in plants and animals, very little is known about the bacterial molecules recognized by such receptors.

Components of innate immune systems in both plants and animals share many conserved features. Most notably, they sense the presence of pathogen-associated molecular patterns (PAMPs), which represent conserved molecular structures, and avirulence (Avr) factors that are strain-specific molecules produced by phytopathogens. Recognition by the host is via cell surface or cytoplasmic receptors ***(2,3)***. These receptors share common protein domains such as leucine-rich repeats (LRRs), which act as ligand recognition domains, and

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

conserved signaling domains, such as Toll-interleukin 1 and serine threonine kinase domains ***(4)***. Naturally occurring mutations of LRR residues that interfere with ligand binding are correlated with several human diseases, including Bernard-Soulier syndrome and Chron's disease ***(5,6)***.

Intracellular recognition of both PAMPs and Avr factors is largely carried out by the cytoplasmic nucleotide-binding oligomerization domain (NOD) protein family. The NOD family contains a large number of proteins from animals, plants, fungi, and bacteria ***(7)***. Genetic variation in three human NOD family members has been implicated in the development of disease ***(7)***. Similarly, variations in plant NOD family members determine levels of resistance to bacterial, fungal, insect, and viral pathogens underscoring the essential role of the NOD-mediated innate immune response in plant and animal biology.

In animals, recognition of PAMPs in extracellular compartments or at the cell surface is largely carried out by the Toll-like receptor (TLR) family that contain LRRs in the extracellular domain and a Toll-interleukin 1 intracellular domain ***(8)***. Although TLRs recognize diverse molecules, they activate a common signaling pathway to induce a core set of defense responses ***(9)***. Several bacterial PAMPs have been identified to date, including flagellin (recognized by TLR5 ***[10]***), lipopolysaccharide (recognized by TLR4 ***[11]***), and a modified peptide (muramyl dipeptide, recognized by Nod1 ***[12]***).

Surprisingly, little is known about how plant hosts sense and respond to PAMPs or Avr factors at the cell surface. The best characterized examples are the tomato CF receptors that detect *Cladosporium fulvum* Avr peptides ***(13)***, the *Arabidopsis* FLS2 receptor kinase (RK) that detects flagellin, a proteinasceous component of bacterial polar flagella, and the rice Xa21 RK that mediates recognition of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains expressing AvrXa21 activity. In this chapter, the term AvrXA21 pathogen-associated molecule(s) (PAM) will be used to designate the molecule(s) produced by *Xoo* that triggers the Xa21-mediated innate immune response. Resistance conferred by the *Xa21* gene is quite broad spectrum, with resistance to 29 of 32 strains tested, suggesting that all 29 strains carry AvrXa21 activity ***(14)***. Whereas plants lacking XA21 are susceptible to most races of the pathogen *Xoo*, *Arabidopsis* plants lacking FLS2 display no disease phenotype ***(15)***, confounding the precise role of FLS2 in disease resistance.

Despite these distinctions, both FLS2 and XA21 carry LRRs in the presumed extracellular domain, are members of large polymorphic gene families (in the case of *Xa21*, at least 40), and fall into a distinct phylogenetic subclass, the LRRXII class (***[16]***; CD and PR, unpublished), suggesting that FLS2 and XA21 mediate recognition of PAMs in a conserved manner. Recently, a rice RK named XA26 that is closely related to XA21 and FLS2 was cloned and demonstrated to confer resistance to *Xoo* (Q. Zhang, personal communication). This

result suggests that many of the approx 1100 largely uncharacterized rice RKs may be involved in PAM perception. Interestingly, like FLS2, at least two other plant LRR–RKs serve as receptors for small peptides, including the presumed receptor for phytosulfokine (a sulfated peptide that plays a key role in cellular dedifferentiation and proliferation in plants), and systemin (a plant signalling molecule *[17–19]*). As is the case with RKs in animals, most plant RK ligands identified so far are secreted peptides *(20)*.

In summary, there is increasing evidence that TLRs, NODs, and plant RKs share conserved recognition and signaling domains, that their signaling pathways are conserved, and that they recognize diverse PAMs from plant and animal pathogens *(15,21)*. Given the importance of these proteins in innate immune recognition and host defense, there is great interest in identifying the PAMs that they detect, elucidating the secretion and modification of these molecules, and determining their role in the biology of the pathogen.

In our laboratory, efforts are underway to identify new genes required for AvrXa21 PAM activity and to determine the product and function of the genes with various molecular techniques. Among them, inactivation of a gene via maker exchange mutagenesis and recovery of the gene via complementation of a mutant are invaluable tools for understanding the physiology and the significance of specific genes in the virulence of pathogens. For the last a few years, we have applied marker exchange mutagenesis using double crossover (DCO) and complementation strategies to understand the function of the *rax* (required for AvrXa21 activity) genes. We have cloned eight *rax*-genes from *Xoo*, which causes bacterial blight disease in rice. We generated nonpolar mutants using the cloned genes and puc18, and complement mutants with the cloned genes and the pUFR027 or pML122 vector *(22–25)*. Through analysis of phenotype changes of the mutants in inoculation experiments, we confirmed that the genes are required for AvrXa21 activity.

2. Materials

1. pUC18 vector.
2. pUC-4K vector (Pharmacia).
3. Restriction enzymes and reaction buffers (NEB).
4. T4 DNA ligase with reaction buffer (NEB).
5. NB medium.
6. PSB medium:10 g of peptone, 10 g of sucrose, 1 g of sodium glutamate for 1 L, pH 7.0).
7. Antibiotics (kanamycin, cephalexin, gentamycin, ampicillin).
8. Spectrophotometer.
9. Cell-Porator™ (BRL).
10. *Escherichia coli* strain DH10B.
11. *Xoo* strain, *PX099*.

12. pET15-b vector.
13. pUFR027 or pML122 vector.
14. TEN buffer: 200 m*M* Tris-HCl, pH 7.5, 1 m*M* ethylene diamine tetraacetic acid, 1 *M* NaCl.
15. TMN buffer: 50 m*M* Tris-HCl, pH 7.5, 50 m*M* $MgSO_4$.

3. Methods

The following protocols are described based on our work for *Xoo*.

3.1. Marker-Exchange Mutagenesis

3.1.1. Vector Construction for DCO Event

1. Construct a plasmid coding the target gene by using multiple cloning sites in a suicide plasmid (*see* **Note 1**), which are not able to replicate in *Xoo* (conditional replicons). The conditional replicon must have a gene encoding a selectable marker for antibiotics resistance. General *E. coli* vector such as a pUC18 has been used for generation of *Xoo* knockout mutants in our laboratory.
2. Disrupt the coding sequences of the target gene with restriction enzyme(s) available for insertion or substitution of a marker gene. An antibiotic resistance marker such as the Kanamycin-resistant gene (*Kan^r^*) or another gene for which there is an easily selected phenotype are generally used as the marker gene (*see* **Note 2**). The marker must be different from the plasmid marker (Ampicillin resistant gene [*Amp^r^*] in pUC18). In this step, homologous fragments for DCO event of your target gene disrupted by the inserted marker would be better to be longer than 400 bp (*see* **Note 3**).
3. Ligate the linearized plasmids and marker genes with T4 DNA ligase at 4°C overnight.

3.1.2. Preparation of Xoo-Competent Cells

1. Grow an overnight culture (OD_{600} = 0.8-1.0) of *Xoo* cells in 40 mL of NB containing cephalexin (25 μg/mL) on a rotary shaker at 28°C.
2. Harvest by centrifugation at 2500*g* at 4°C for 10 min.
3. Suspend the cell pellet with 15 mL of cold TEN buffer by pipetting.
4. Repeat **steps 2** and **3** three times.
5. Centrifuge at 2500*g* at 4°C for 10 min.
6. Resuspend with 15 mL of TMN buffer by pipetting.
7. Chill on ice for 2 h.
8. Repeat **steps 5** and **6**.
9. Suspend with 15 mL of cold DDW by pipetting
10. Harvest the cell with centrifugation at 2500*g* at 4°C for 10 min.
11. Suspend with 15 mL of cold 15% glycerol–water solution.
12. Transfer 20 μL of cells to 0.5-mL tubes on ice.
13. Stock in –80°C freezer

3.1.3. Electroporation of the Construct Into Xoo*-Competent Cells*

1. Mix 20 µL of *Xoo*-competent cells and 1 to 2 µL (10 ng) of recombinant plasmids.
2. Transform by using electroporation (Cell-Porator™: 700 V, 4KΩ).
3. Transfer the cell to 1 mL of liquid PSB medium and culture for 2 to 3 h at 28°C.
4. Plate the cells onto PSA medium plates that contain the appropriate antibiotics (Kanamycine: 50 µg/mL) for selection of mutants, and incubate at 28°C.

3.1.4. Selection of the Mutant by DCO Event

1. Plate the putative mutants from PSA plate containing 50 µg/mL of kanamycin on PSA containing kanamycin and kanamycin (50 µg/mL) / ampicillin (100 µg/mL), respectively.
2. Incubate at 28°C for 2 or 3 d.
3. Select mutants grown on PSA plate containing kanamycin (50 µg/mL), not on PSA plate containing kanamycin (50 µg/mL) / ampicillin (100 µg/mL; *see* **Notes 4** and **5**).
4. After selection on replica plates, the marker exchange event can be confirmed by Southern blot analysis (*see* **Note 6**) or colony polymerase chain reaction.

3.2. Complement and Overexpression Mutant

3.2.1. Vector Construction

1. Clone your favorite gene into the pET-15b vector by using available cloning site. Using this cloning step, six sequential copies of Histidine are fused to N-terminus of the coding sequences. This His-tag from pET-15b will be feasible for confirmation of the gene expression in the target cells with Western blot analysis (*see* **Note 7**).
2. Excise the fused fragment from the construct by using available restriction enzyme(s) for cloning to expression vector. We have used pML122 or pUFR027 (*see* **Note 1**).
3. Ligate the gene fused by six histidines and vectors (pML122 or pUFR027) with T4 DNA ligase at 4°C overnight.

3.2.2. Introduction of the Construct Into Xoo*-Competent Cells*

1. Introduce pML122 carrying the His-tag fused gene into *Xoo*-competent cells (*see* **Subheading 3.1.2.**) in which target gene expression was inactivated by marker exchange mutagenesis by using electroporation (Cell-Porator: 700 V, 4KΩ).
2. Transfer the cell to 1 mL of liquid PSB medium and culture for 2 to 3 h at 28°C and then plate onto PSA plates containing kanamycin (50 µg/mL)/gentamycin (15 µg/mL; *see* **Note 8**).
3. Confirm the transformant with isolated plasmids, and Western blot analysis with His-antibody for expression of the gene (*see* **Note 6**).
4. Stock in –80°C freezer (*see* **Note 9**)

4. Notes

1. A narrow host range vector for *E. coli* can be used as a suicide vector for *Xanthomonas* broad host range vectors, such as pML122 and pML123 ***(26)***, or pUFR027 and pUFR034 ***(27)***, which replicate in *Xanthomonas* and cannot be used. pML122/123 uses pML10 as the template vector and contains two selective marker genes (*Kan*^{r}, *Gm*^{r}) and the promoter of the *Nm*^{r} gene. The vectors (pUFR027 and 034) contain the pSa origin of DNA replication, *parA* from the *Agrobacterium* plasmid pTAR, neomycin-resistant gene as a selection marker, and a *lacZ* cassette with cloning sites.
2. In our laboratory, the *Kan*^{r} gene from pUC-4K (Pharmacia) or the *Spec*^{r} gene from the TOPO have both been used for the marker. In the case in which a double gene knockout mutant is being generated, two different selective markers are needed.
3. We recommend using more than 400 bp for the DCO event, but it is not impossible to cause the DCO event with shorter DNA fragments. However, the efficiency of the DCO event is considerably lower with shorter DNA fragments.
4. The putative mutants from kanamycin plates might have both (DCO and single crossover [SCO]) mutants, but the DCO mutants can be selected by replica plating (kanamycin and kanamycin/ampicillin). DCO mutants carry only the *Kan*^{r} gene used for disruption of the target gene, whereas SCO mutants contain both the *Kan*^{r} gene and the plasmid marker gene (*Amp*^{r}) in the *Xoo* genome. This selection step is important because if the homologous regions for recombination include sequences 5'- or 3'- to the coding portion of the target gene, SCO events can recreate a complete gene and DCO mutagenesis will be unsuccessful.
5. In some cases, a direct screen for DCO is not feasible because DCO events that incorporate a gene from a plasmid into the chromosome are infrequent. In this case, a two-step method is used. Although the SCO mutants carry the entire plasmid containing both the mutant and wild-type copies, the wild-type copy can be removed by second recombination event between the flanking direct repeats through succeeding a generation.
6. The standard technique for southern and western blot analyses is used ***(28)***.
7. If you have other methods to detect expression of your gene, you don't need to use pET15-b and start from **step 3**. In some case, the six histidines at the N-terminus can change conformational structure of protein and, therefore, the biological function of the protein could be lost.
8. Growth of the transformant could be slow or unsuccessful on selection medium containing two antibiotics (kanamycin and gentamycin) because pML10, the template vector for pML122/123, has different copy numbers in different species (45, 70, 105, 45 copies in *E. coli*, *Pseudomonas putida*, *Rhizobium melitoti*, and *Rhizobium leguminosarum*, respectively) and the copy number is much lower than other *E .coli* vectors. In this case, you can select for transformants using one half the concentration of antibiotics (25 μg/mL of kanamycin and 7.5 μg/mL of gentamycin) or you can use a two-step selection, with kanamycin followed by gentamycin.

9. Safekeeping of the transformants carrying pML122 constructs in –80°C are important, because the vector is not stable and has low copy number. To obtain accurate results with the transformants, it would be better to use the fresh cells from stock.

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3

Whole-Genome Analysis to Identify Type III-Secreted Effectors

Boris A. Vinatzer and Jean T. Greenberg

Summary

Many Gram-negative plant and animal pathogens share a common virulence strategy that relies on the specialized type III secretion system. This apparatus is used to secrete virulence factors, called effectors, into the extracellular host environment and directly into the cytoplasm of host cells. Effectors interfere with host signaling and host metabolism to create an optimal environment for pathogen replication. The identification of effectors in plant pathogens was limited for many years to those effectors that elicit strong plant defenses on some hosts. The members of this subset, called avirulence proteins, can be readily identified because they dominantly confer strong defense-inducing properties to a heterologous virulent strain. This chapter describes two methods to identify type III-secreted effectors in plant pathogens independently of their phenotype. The first method consists of an in vivo molecular genetic screen that uses the activity of an avirulence protein to identify effectors without avirulence activity. It should be possible to apply this method to most Gram-negative plant pathogens. The second method consists of a bioinformatic approach applicable to those pathogens for which at least a draft genome sequence is available.

Key Words: *Pseudomonas syringae*; type III secretion; TTSS; effectors; avirulence; *hrp* box; transposon; *avrRpt2*; *Arabidopsis thaliana*.

1. Introduction

Most important Gram-negative plant pathogens are extracellular and rely on a type III secretion system (TTSS) to secrete proteins into the extracellular host environment and directly into the host cytoplasm. Pathogens that are deficient in type III secretion are unable to grow *in planta* or to cause disease, suggesting that the proteins secreted by the TTSS are essential virulence factors. Some authors make a distinction between type III-secreted proteins that are predicted—based on their predicted enzymatic activity—to be targeted to the extra-

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

cellular host environment and those that are targeted to the host cytoplasm. They call the first kind "helper proteins" and the latter ones "effectors" ***(1)***. Because no evidence exists thus far for differential secretion of type III-secreted proteins in plant pathogens, all type III-secreted proteins will be called effectors in this chapter.

There is one prominent group of effectors of plant pathogens that have a striking phenotype: they can dominantly confer to virulent pathogens the inability to cause disease. On certain hosts, these effectors induce a resistance response that is usually accompanied by a type of programmed cell death called the hypersensitive response (HR ***[2]***). When the dose of the bacterial inoculum is high enough, the HR is macroscopically visible as a total leaf collapse and can easily be scored by eye in controlled infections. Because such effectors turn a virulent pathogen into one that is "avirulent," these effectors are called "avirulence" (Avr) proteins encoded by avirulence (*avr*) genes. An individual Avr protein usually is recognized by an individual plant Resistance protein coded for by a resistance (*R*) gene that segregates as a single locus. The concept of cognate avr–R pairs is known as the "gene-for-gene" relationship and was first described by Flor ***(3)***. The first *avr* genes were identified by constructing genomic DNA libraries of a strain avirulent on one host and transforming this library "en masse" into a virulent strain on the same host. Avirulent transformants were subsequently screened by individual inoculations on plants. Library clones that conferred avirulence were sequenced and the individual *avr* gene was identified. Among others, this clever technique allowed the molecular identification of the first *avr* gene ***(4,5)***.

Other effector genes were identified by their proximity to the gene cluster coding for the TTSS components. Any mutation in a TTSS component important for the actual secretion process eliminates the ability of a pathogen to cause disease in the case of a virulent pathogen and to elicit an HR in the case of an avirulent pathogen. The genes coding for TTSS components are therefore called *hrp* (HR and pathogenicity) genes. The *hrp* genes are always clustered and are localized either on the bacterial chromosome or on a plasmid. In *Pseudomonas syringae* the *hrp* cluster is flanked on both sides by effector genes ***(6)***. On one side, the effectors are conserved among many *P. syringae* strains and this cluster was therefore called the conserved effector locus (CEL). The other side of the TTSS contains effectors that are not as well conserved among strains and was therefore called exchangeable effector locus (EEL). Note that although some of the genes in the CEL and EEL are *avr* genes, others are not and that not all *avr* genes are located in the CEL or the EEL.

Effectors in the animal pathogen *Yersinia* and some other animal pathogens are efficiently secreted into the culture medium under certain conditions. None

of the plant pathogens efficiently secretes effectors in culture, but *P. syringae* secretes some effectors in sufficient amounts in culture to be sequenced and some in fact were. Yuan and He ***(7)*** identified HrpA, a structural component of the TTSS, and HrpZ by sequencing proteins from the culture supernatant of *P. syringae*. Because many effectors are not secreted efficiently in culture, this approach is limited.

Individual effector knockouts in plant pathogens usually have subtle effects on virulence. Why this is so has not been answered satisfactorily yet, but it is believed to be mainly to the result of redundancy between effectors. This chapter describes two methods for identifying effectors in plant pathogens independently of any knowledge about their Avr activity, location, ability to be secreted in culture, or knockout phenotype. The first method is based on two findings:

1. The AvrRpt2 effector has two distinct regions, an N-terminal region that is important for secretion, and a C-terminal effector region that harbors the avirulence activity and that is sufficient to induce an HR in *Arabidopsis thaliana* upon recognition by the plant R protein Rps2 ***(8,9)***.
2. The effector region of AvrRpt2 can be secreted from *P. syringae* when fused to the heterologous secretion region of the AvrRpm1 effector ***(9)***.

On the basis of these two findings, we developed an in vivo screen using the effector region of AvrRpt2 as reporter ***(10)***. We constructed a minitransposon that carries an origin of replication for *Escherichia coli*, an origin of transfer, and the tn5 *tranposase* gene on the vector backbone. The DNA coding for the effector region of AvrRpt2 (amino acids 81–255) and an antibiotic resistance marker are located between the tn5 insertion sequences. This construct can be transferred from *E. coli* to *P. syringae* by triparental mating. When the individual construct enters an individual *P. syringae* cell, the transposase is activated and can insert the DNA coding for AvRpt2$^{81–255}$ and the antibiotic resistance marker randomly into the *P. syringae* genome. Because the construct has no origin of replication functional in *P. syringae*, it is lost after cell division. Furthermore, because the tranposase gene is not included between the insertion sequence elements, it is lost together with the construct and a stable insertion line is created. When by chance the minitransposon is inserted in-frame downstream of the secretion region of an effector gene, a fusion between this secretion region and AvRpt2$^{81–255}$ is created. The resulting fusion is secreted into plant cells where it elicits an HR upon the interaction between AvrRpt2$^{81–255}$ and Rps2. Because this HR is very strong, one HR-eliciting strain mixed with seven non-HR eliciting strains is enough to cause leaf collapse. Pools of eight insertion strains can therefore be infiltrated into leaves and positive pools can then be deconvoluted to identify the “culprit.”

Once an HR-eliciting strain is identified, the sequence of the gene into which the transposon inserted has to be determined. Then, the TTSS-dependent secretion of the fusion, and the Rps2 dependence of the HR have to be verified. These controls are needed to rule out the possibility that the fusion is secreted through a different kind of secretion system and that the observed cell death is caused by the toxicity of the fusion product and not by recognition of $AvRpt2^{81-255}$ by Rps2. We anticipate that instead of the AvrRpt2 reporter in *P. syringae* infections of *A. thaliana*, any other effector of any plant pathogen that elicits a strong HR on any plant could be used in a similar screen.

The second method for effector identification described in this chapter consists of a bioinformatic approach to effector prediction. It is based on the fact that effectors in *P. syringae* and in other plant pathogens have amino acid biases that distinguish them from other proteins and that in many plant pathogens effector genes are preceded by conserved sequences in their promoters ***(11)***. In the case of *P. syringae*, effector proteins are richer in serine than noneffector proteins and effector genes (or operons) are preceded by the conserved *hrp*-box promoter element as reviewed in **refs. *1*** and ***11***. Other plant pathogens have similar biases and similar or different promoter elements. *Erwinia amylovora* effectors for example have also *hrp*-boxes, whereas *Ralstonia solanacearum* and *Xanthomonas* sp. effectors are preceded by a so-called PIP box, also called hrp_{II} box ***(12,13)***. Once effector candidates have been identified by this approach, they can be validated using the AvrRpt2 reporter, for example.

2. Materials

2.1. Mating the Minitransposon Into P. syringae

1. Bacterial strains: *E. coli* VPE42 (kan^r, tet^r) containing the mini-transposon vector pDSG50 (amp^r, kan^r) or similar mini-transposon vector, helper strain *E. coli* RK600 (cm^r; *see* **Note 1**), a *P. syringae* strain of choice and a TTSS-deficient strain, derivative of the same (*see* **Note 2**).
2. LB medium: 1 L of water, 10 g of bacto-tryptone, 5 g of bacto-yeast extract, and 10 g of NaCl.
3. KB medium: 1 L of water, 10 g of bacto-proteose peptone, and 1.5 g of K_2HPO_4. After autoclaving and cooling to at least 65°C add 3.2 mL of autoclaved 1 *M* $MgSO_4$ and 25 mL of autocolaved 20% glycerol.
4. All agar plates contain 15 g/L of agar.
5. Antibiotics: streptomycin, kanamycin, and nitrofurantoin (toxic; *see* **Note 3**).
6. For replica plating, a commercial or homemade "replicator" is used in combination with velvets (available from fabric shops or from laboratory supply companies).

Table 1
Sequences of Primers Used

Primer name	Sequence
p1	CCTTTGTTCCGTCTCACGCACGTTC
p2	GGAATCGGAAGCCACGCTCGAACTATC
p3	CGGCCGCACTTGTGTATAA
p4	TAATTCCGCGAACCCCAGAG
p5	CGGCCTAGGCGGCCAGAT
p6	GAAGGCGATAGAAGGCGATG
v1	GAGAGGCGGTTTGCGTATTG
v2	ATGCTTCCGGCTCGTATGTT

2.2. Plant Growth

1. Potting soil.
2. Seed of *A. thaliana* ecotype "Columbia" and the *rps2* mutant line is available from http://www.arabidopsis.org/servlets/TairObject?id=1005161473 &type= germplasm.

2.3. Plant Infections and HR Evaluation

1. Toothpicks (*see* **Note 4**).
2. *P. syringae* mini-transposon insertion strains are grown in 96-well growth blocks with 2-mL wells (reusable if bleached and washed after each use).
3. $MgSO_4$ is autoclaved as a 1 *M* stock solution and diluted when needed in sterile water to 10 m*M*.
4. 1-mL Blunt end syringes for plant infections are available from medical or laboratory supply companies.

2.4. Sequencing Flanking Regions

1. Glycerol.
2. Cryogenic vials.
3. Restriction enzymes (*Bsa*AI, *Bsp*1286I, *Fsp*I, *Msp*I, *Nco*I, and *Sac*I when using pDSG50) and a thermostable polymerase.
4. Primers for inverted polymerase chain reaction (I-PCR) when using pDSG50 are listed in **Table 1**.
5. Sequencing of PCR products can be outsourced to a sequencing center.
6. Custom primers can be ordered from many biotech companies.
7. Agarose, TBE, or TAE buffer.

2.5. In Vivo Effector Verification (Type III Dependence Test)

1. Emzymes for PCR amplification and ligation can be purchased from any major molecular biology company.

2. The plasmid pBAV208 is available from the authors.
3. Electro-competent or chemical-competent *E. coli* DH5α cells.

2.6. In Silico *Screening*

1. Bioperl software available as a free download from www.bioperl.org.
2. "Amino acid bias" script written by Gregory Kettler downloadable from http://preuss.bsd.uchicago.edu/index3.html?content=aascreen.html for free.
3. Any web browser to access online databases.

3. Methods

We describe here how to use the minitransposon pDSG50 that carries the AvRpt2$^{81\text{-}255}$ reporter in *P. syringae* on *A. thaliana*. This construct was used in the effector screen described in Guttman et al. ***(10)***. We describe in **Note 5** how to substitute the AvRpt2$^{81\text{-}255}$ reporter with other reporters to apply the in vivo screen to other pathogens on other plants.

3.1. Mating the Minitransposon Into P. syringae *(see Note 6)*

1. Grow the *E. coli* strain VPE42 containing pDSG50, the helper strain *E. coli* RK600 and the receiving *P. syringae* strain separately overnight at 30°C in 5 mL of liquid medium each (using LB for the *E. coli* strains and KB for *P. syringae* plus respective antibiotics).
2. Dilute the *E. coli* cultures 1:50 in the morning and *P. syringae* 1:10. When the *P. syringae* culture has reached an OD_{600} of approx 1.0 (this is after 3 to 5 h depending on the *P. syringae* strain used), spin down 1 mL of each of the cultures at 2000*g* in a tabletop microcentrifuge for 5 min.
3. Resuspend each of the pellets in 100 μL of sterile 10 m*M* $MgSO_4$ by vortexing at medium speed, combine the three strains in one tube, and vortex at medium speed for a few seconds.
4. Spread the mixture on KB plates without antibiotics so that the strains can mate. Always plate a series of different volumes because the mating efficiency is pretty variable. Plate 10, 50, and 100 μL (each volume is spread on several plates to obtain at least 800 well-separated colonies in total).
5. Plates have to be incubated for approx 1 d at 28 to 30°C.
6. Replica plates onto KB plates with selection for *P. syringae* (streptomycin in the case of *Pma*ES4326; *see* **Note 7**) and for the transposon (kanamycin in the case of pDSG50) using a replicator (*see* **Note 8**).
7. After another 2 to 3 d at 28 to 30°C colonies should become visible and after another day they should be big enough to be picked into liquid cultures for plant infections (*see* **Note 9**).

3.2. Plant Growth

1. Soak *A. thaliana* ecotype "Columbia" seed in 0.1% agar.
2. Vernalize the seed for 3 to 10 d at 4°C (*see* **Note 10** for hints on planting and growing *Arabidopsis*).

3. Plant seeds using a Pasteur pipet and rubber bulb placing individual seeds on the surface of wet soil.
4. Plants are grown under long day conditions (16 h light) at 20°C and 50% humidity. The plants are ready for infection when they are between 3 and 4 wk old (this corresponds to the week that precedes bolting).

3.3. Plant Infections and HR Evaluation

1. Fill a 96-well growth block that has 2-mL large square wells with 1 mL of KB medium per well.
2. Using toothpicks, pick up as many bacteria as you can from each colony obtained in the matings (*see* **Note 11** for hints on growing *P. syringae* in 96-well growth blocks).
3. Use also one colony containing *P. syringae* expressing full-length AvrRpt2 as positive control in one well of one block for each eight blocks you fill.
4. Take the toothpicks out and grow the blocks in a shaker at 30°C for approx 20 h at the highest speed possible that does not cause the cultures to spill from well to well.
5. After 20 h the cultures should be saturated. You now prepare a growth block containing 1 mL of 10 m*M* $MgSO_4$/well.
6. Measure the average OD_{600} of your overnight cultures
7. Using a multichannel pipet, add to each row of the 10 m*M* $MgSO_4$ block an entire *P. syringae* growth block combining the 8 rows of one entire block with overnight cultures of *P. syringae* into one row of the $MgSO_4$ block (*see* **Note 12**). Add as much culture as needed to obtain a final OD_{600} between 0.3 and 0.5. This corresponds more or less to adding 5 to 10 µL of each of eight saturated cultures (40 to 80 µL total) to 1 mL of $MgSO_4$.
8. Keep the overnight growth blocks until the next day (room temperature is fine). You need the individual cultures in order to identify any individual positive colony of a possible HR eliciting positive pool the next day!
9. Infect one block of eight pooled overnight growth blocks on one flat of 96 plants. Infiltrate the three largest leaves/plant with a blunt-end 1-mL syringe pressing the syringe against the lower side of the leaf after marking the leaves you chose to infiltrate with a marker pen. Do not water the plants between infection and scoring.
10. Sixteen to eighteen hours later, score the plants for an HR (*see* **Note 13**).
11. If a plant looks like it has an HR (**Fig. 1**), go back to the corresponding growth block and stamp the eight cultures forming the putative positive pool onto a KB plate containing the appropriate antibiotics.

3.4. Identification and Verification of Positive Insertion Strain

1. If only one or a few putative positive pools were obtained, you can test the putative individual positives by growing them up individually in 5 mL of KB in 15-mL test tubes and infecting them individually on plants. If there are many putative positive pools, the individual strains composing the positive pools can be grown up again in a growth bock and then be reinfected in pools of four. If a pool of four is positive, the individual strains forming that pool should then be tested individually.

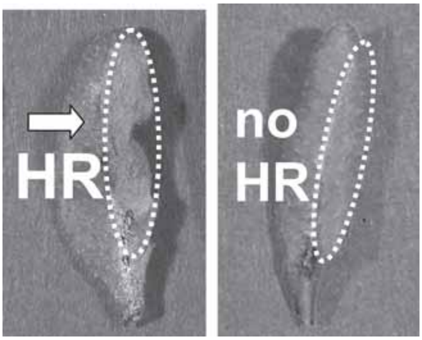

Fig. 1. Leaf of the *Arabidopsis thaliana* ecotype "'Columbia" 18 h after infiltration with a hypersensitive response (HR)-eliciting and with a non-HR-elicting *Pseudomonas syringae* strain. The area infiltrated with bacteria is indicated with a dotted line.

2. Once you have identified an individual HR eliciting strain, it must be streaked out to a single colony and a few single colonies are tested again on plants to confirm the individual positive colony. At this point, *rps2* mutant plants should also be infected to make sure that the positive strain elicits an *RPS2*-dependent HR is caused by the recognition of the reporter and not by the toxicity of the fusion product.

3.5. Sequencing Flanking Regions

1. Once an individual positive colony has been isolated, it is grown up overnight to saturation.
2. Make a glyercol stock adding 300 μL of sterile 50% glycerol to 700 μL of saturated culture and store in a cryogenic vial at –75 to –85°C.
3. Extract genomic DNA using the Gentra Puregene kit or similar kit from other suppliers.
4. Dilute the genomic DNA to a final concentration of 50 ng/μL to use it for I-PCR (*see* **Note 14** and **Fig. 2**).
5. Use 2 μL of DNA in a total reaction volume of 20 μL to digest DNA for at least 2 h. When using pDSG50 digest DNA separately with *Bsa*AI, *Bsp*1286I, *Fsp*I, *Msp*I, *Nco*I, and *Sac*I (*see* **Note 15**).
6. Inactivate the restriction enzymes by heating the reactions at 80°C for 10 min.
7. Use 3 μL of the reactions in a ligation reaction in a 20-μL volume using 1 μL of ligase.
8. Incubate overnight at 15°C.
9. Use 2 μL of the reactions as template for PCR using primers on the mini-transposon pointing away from each other (**Fig. 2** and **Table 1**). Use primers p1 and p2 for ligated *Bsa*AI, *Fsp*I, *Msp*I, and *Sac*I digests and use primers p4 and p5 for ligated *Bsa*AI, *Bsp*1286I, *Msp*I, and *Nco*I digests. All primers work well with a 58°C annealing temperature.

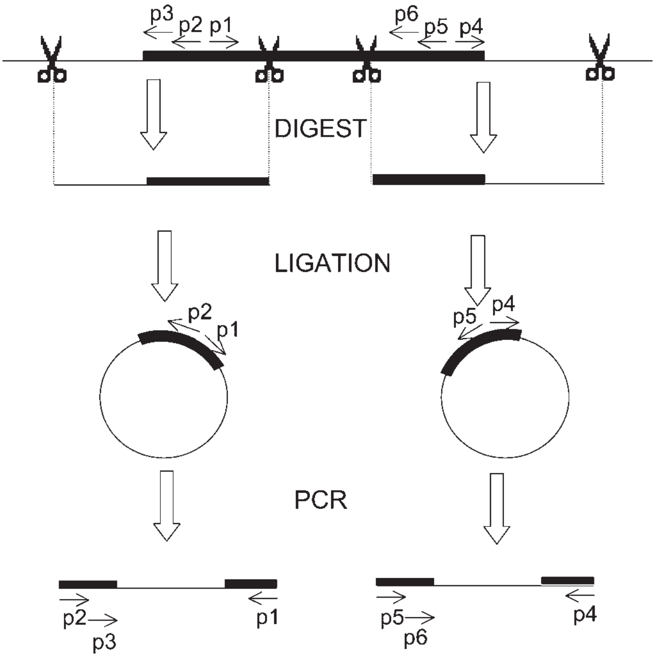

Fig. 2. Inverted polymerase chain reaction schematic.

10. Run PCR products on a 0.8% agarose gel in 1X TBE.
11. If at least one reaction gives rise to a clean individual band of at least 500 bp, cut the band from the gel, clean it (using a commercial kit) and have it sequenced using primers p3 and p6, respectively (**Fig. 2** and **Table 1**). *See also* **Note 16** for more advice on troubleshooting I-PCR.

3.6. In Vivo Effector Verification (Type III-Dependence Test; See *Note 17)*

1. Design a primer approx 500 bp upstream of the insertion site pointing toward the minitransposon and use this primer in combination with the primer p5 (**Table 1**) in a PCR using Pfu Turbo or Ultra (Stratagene, La Jolla, CA) or another high-fidelity polymerase (*see* **Note 18**).
2. Amplify pBAV208 with the primers v1 and v2 (**Table 1**). This is your vector fragment.
3. Run PCR products on a 0.8% agarose gel.
4. Clean the bands corresponding to your PCR products of **step 1** and **2** using Quiagen PCR purification kit or similar kit from other suppliers.
5. Phosphorylate the PCR product obtained in **step 1** with T4 polynucleotide kinase (PNK) by resuspending your cleaned DNA in 30 µL of water or Tris-HCl pH 8.5 and adding 3.5 µL of a 10X ligase buffer and 1.5 µL of T4 PNK.
6. Incubate for 30 min at 37°C.

7. Clean the phosphorylation reaction using Quiagen PCR purification kit or similar kit from other supplier.
8. Ligate phosphorylated PCR product from **step 5** with cleaned PCR product from **step 2** using a molar ration of 5 to 1 and incubating overnight at room temperature.
9. Transform *E. coli* DH5α with your ligation and select for kanamycin-resistant colonies.
10. Extract DNA and verify the correct insert using primers on your gene.
11. Mate the resulting constructs back into the wild-type *P. syringae* strain used in the screen and into a TTSS-deficient derivative as described in **Subheading 3.1.** (*see* **Note 19**).
12. Infect two colonies from each mating, your wild-type strain and the original insertion strain on plants. If the fusion gives an HR only when expressed in the wild-type strain, but not when expressed in the TTSS-deficient strain, the fusion is secreted by the TTSS (*see* **Note 20**).

3.7. In Silico *Screening*

*3.7.1. Identification of Open Reading Frames With an Amino Acid Bias (*see ***Note 21****)*

1. If you do not have your own sequence data, download the sequence files from public databases in which you want to search for effector candidates.
2. Save the sequences as simple text files.
3. Download the bioperl software from www.bioperl.org and the amino acid script from http://preuss.bsd.uchicago.edu. You enter the name of the script, the search parameters and the name of your sequence file in the program line following the instructions given on the webpage. In the case of *P. syringae* you look for at least 6 serines in the first 50 aa (*see* **Note 22**).
4. In the case of *P. syringae*, also look for the absence of aspartate in the first 15 amino acids of the proteins you identified because aspartate is rarely found in this region.

3.7.2. Open Reading Frame Verification and Open Reading Frame Finding on Opposite Strand

1. Verify that the predicted effectors could actually be real genes by looking for open reading frames (ORFs) on the opposite strand. If there is an ORF on the opposite strand with homology to a known gene, your serine rich ORF is probably not a gene. Do this using the ORF finder at http://www.ncbi.nih.gov/gorf/gorf.html.
2. Look if there is a more likely START codon upstream or downstream of the predicted serine rich ORF. You do this by looking for Shine Dalgarno sequences (*see* **Note 23**).
3. Do a blastp search with your predicted effector at www.ncbi.nih.gov/BLAST/. If there is a confirmed effector homologous to your predicted effector, the START of the homolog is probably the START of your predicted effector (*see* **Note 24**).

Table 2
***hrp* Box Sequences**

hrp Box consensus
GGAACC 15/16N CCAC
GGAACT 15/16N CCAC
GGAATT 15/16N CCAC
GGAACC 15/17N ACAC

4. In the blastp search also look for homology of your predicted effector to eucaryotic genes. If you find such homology, this is supporting information that your predicted effector acts inside the eukaryotic host cell.
5. In the blastp search also look for homology to bacterial proteins with a known function inside the bacterial cell. If you find such homology, you probably do not have an effector although it is serine rich. This is especially true when homology extends through the N-terminus.

3.7.3. hrp *Box Identification*

1. In the case of *P. syringae*, you now look for the *hrp* box promoter element. The *hrp* box has one of the sequences listed in **Table 2** and is most likely located between 30 and 200 bp upstream of the START of your effector (*see* **Note 25**).
2. It is relatively straightforward to look for *hrp* boxes by eye or by simply searching for either of the two conserved sequences that make up the *hrp* box using the search function of your sequence or text editor of choice and then looking for the other half of the *hrp* box at a distance of 15 to 16 nucleotides by eye.
3. Look if your *hrp* box is within a known gene on either strand. If this is the case, your *hrp* box is most likely spurious (*see* **Note 26**).

3.7.4. Effector Verification

Once you have a predicted effector you can clone it and fuse it to your reporter of choice. You can clone your predicted effector either using restriction enzymes or using the Gateway recombinational cloning system (Invitrogen, Carlsbad, CA). With the latter method, you can take advantage of a series of Gateway compatible cloning vectors for effector confirmation and characterization that are available from the authors of this chapter.

4. Notes

1. *E. coli* VPE42 is a derivative of the broad host range RP4 conjugal donor strain *E. coli* SM10 *lambda pir*. Other *lambda pir* derivatives can be used if different drug resistances are required, for example S17-1 (strep^r, spec^r). *E. coli* RK600 is a so called helper strain and provides *mob* and *tra* genes for mobilization and transfer of the mini-transposon to *P. syringae*.

2. *P. syringae* pv *maculicola* (*Pma*) ES4326 (Strep^r) was the *P. syringae* strain used in Guttman et al. ***(10)***. This chapter describes the in vivo screening method when using *Pma*ES4326. Other *P. syringae* strains and even other species can be used for the screen as well (*see* **Note 5**). A type III secretion-deficient derivative of *Pma*ES4326 is available from the authors.
3. Most *P. syringae* strains are resistant to nitrofurantoin at 100 µg/mL (toxic). A nitrofurantoin stock solution is made in dimethylsulfoxide at 100 mg/mL and stored at –20°C. Nitrofurantoin stock solution is added to KB agar before pouring plates. It is important to stir KB after adding nitrofurantoin for another minute or two since it does not dissolve immediately.
4. Toothpicks can be reused many times when autoclaved after each use.
5. Tn5 transposons are functional in all Gram-negative bacteria. The pDSG50 minitransposon can therefore be used in any plant pathogen. The *avrRpt2* reporter sequence can be changed to other reporters. pDSG50 is derived from the pBSL118 minitransposon ***(14)*** by cloning *avRpt2*$^{81\text{-}255}$ into the multiple cloning site and then removing 5' upstream sequence because a STOP codon was present upstream of and in frame with *avRpt2*$^{81\text{-}255}$. We can provide you with pDSG50 and the DNA sequence up- and downstream of *avRpt2*$^{81\text{-}255}$ so that you can replace *avRpt2*$^{81\text{-}255}$ with your reporter of choice or you can request the original minitransposon with a series of useful cloning sites from the Netherlands Culture Collection of Bacteria at www.cbs.knaw.nl/databases/index.htm with the catalog number 3379. Any reporter has to be well characterized before use. Make sure that the reporter is sufficient to elicit an HR inside the plant cell. Create defined effector::reporter fusions to make sure fusions to your reporter elicit a strong HR on your host plant. Also perform setup experiments to identify the optimal infection dose, plant cultivar/ecotype, age of plants, and growth conditions to reach the highest possible sensitivity in your screen. It is also useful to add an epitope tag to your reporter if you do not have an antibody against it (*see* **Note 9**).
6. The vector pDSG50 or similar minitransposon vector can be transferred efficiently to *P. syringae* by triparental mating. Because of its RP4 origin of replication, pDSG50 can only replicate in *lambda pir* strains like *E. coli* VPE42, but not in DH5alpha, and needs a helper strain like *E. coli* RK600 to provide mobilization and chromosomal transfer genes (*tra* and *mob*) to be mobilized and transferred to *P. syringae*.
7. In case your *P. syringae* strain is rifampicin-resistant or has no antibiotic resistance, use nitrofurantoin as selection for *P. syringae*. *E. coli* easily acquires spontaneous rifampicin resistance and rifampicin is therefore useless as a selective marker in matings.
8. Wash velvets immediately after each use in water and soap, rinse in water, let dry, and autoclave for 30 min.
9. An efficient mating leads to more than 100 colonies per plate. You should make sure that at least some of the colonies actually produce fusion proteins by doing Western blots on a few dozen insertion strains using an antibody against your reporter (if available) or against an epitope tag that you should have fused to your reporter (*see* **Note 4**). If the genome of the bacterial strain you are using were

100% transcribed, you would expect one in six strains to produce a fusion protein. Because there are non-coding regions and not all genes are expressed in culture medium, you can expect approx 1 in 24 strains to produce a fusion with your reporter. It is also useful to mate at least once a full-length reporter under the control of its own promoter into your strain and to make sure that colonies obtained from this mating give a strong HR (we can provide the construct pDSG49 containing full-length *avrRpt2* under the control of its own promoter for this purpose).

10. Do not use seed that has been vernalized longer than 2 wk because the germination rate will decrease after 2 wk and even the seedlings that look normal at germination may turn red later and not give a good HR. Plants can be grown in a 96-well grid on standard flats containing 48 cells planting two plants diagonally in each cell. To make sure to have 96 plants you can plant four seeds per insert and then thin out to two plants after 2 wk. Make also sure plants are not under or overwatered while growing. Only "happy" plants give a good HR.
11. Use as much inoculum as you can. *P. syringae* is not *E. coli* and a nonvisible amount of bacteria will take for ever to grow. Use a blob of cells of at least 1 mm in diameter to start your culture with. You cannot use too much. If even after using a good-sized inoculum, *P. syringae* cultures do not saturate within 20 h, it is most likely because of a too-slow shaking speed. Also, residual bleach in the wells may interfere with growth. To attach the growth blocks to your shaker, you can build your own growth block holder. Screw a sturdy cardboard box to the shaking platform and squeeze Styrofoam blocks between the growth blocks and the box to keep the growth blocks from moving around. Always cover growth blocks with plastic lids and tape the lids to the blocks or fasten with rubber bands.
12. If some bacteria accumulated at the bottom of the wells, vortex until they resuspend completely before pipetting. You do not have to change tips between samples since the small quantity of cross-contamination will not give you false-positives.
13. The best time to score the HR will depend on your plant growth conditions, your *P. syringae* strain, and the OD_{600} of the inoculum. The first few times you do the screen check the plants several times between 16 and 24 h. The best time to score the HR is when the plants infected with negative pools still look healthy but are about to wilt. Note that positive pools may give a weaker or a stronger HR than the pool containing the positive control.
14. I-PCR is a technique that allows you to obtain PCR products of unknown genomic regions flanking a known DNA sequence, in our case the unknown sequences flanking the transposon insertion.
15. The enzymes *Bsa*AI, *Fsp*I, *Msp*I, and *Sac*I cut pDSG50 downstream of the primer sites p1 and p2. These digests will be used to obtain PCR products to sequence the region upstream of the transposon insertion. The enzymes *Bsa*AI, *Bsp*1286I, *Msp*I, and *Nco*I cut the minitransposon upstream of the primer sites p4 and p5. These digests will be used to obtain PCR products to sequence the region downstream of the insertion site. When using a minitransposon different from pDSG50, you have to find restriction sites in similar positions regarding to the primer sites you will use.

16. If you obtain a strong band with a background smear or additional weaker bands, the strongest band should be cut out, cleaned, and diluted 1:100 (you may try a dilution series of 1:10 to 1:100) and used as template for a second PCR reaction using the forward primer from the first PCR with a more internal second primer p3 or p6 (*see* **Fig. 2**). A 2-μL aliquot of this PCR should be run on a gel and if a clean band is now obtained the rest of the PCR can now be cleaned and sequenced. If no bands are obtained with any restriction enzyme, a different polymerase can be used. Pfu or other similar high-fidelity enzymes can amplify longer DNA fragments compared with Taq. Thus, using such polymerases increases the chance of getting an I-PCR product. In any case I-PCR may not lead to the identification of the complete effector and promoter sequence of every effector found in the screen. If this is the goal, a genomic library of the strain used in the screen should be constructed and hybridized to the DNA fragments obtained by I-PCR. Positive clones can then be sequenced to extend the sequences surrounding the transposon insertions.
17. To confirm that the identified effector::AvRpt2$^{81\text{-}255}$ fusions are secreted in a TTSS-dependent manner, you amplify by PCR at least 500 bp of the DNA sequence upstream of the insertions together with the AvrRpt2 reporter and the kanamycin resistance gene of the minitransposon and clone this whole fragment into a high-copy number cloning vector that has no origin of replication for *P. syringae*.
18. Pfu creates blunt DNA fragments that are unphosphorylated. A Pfu product that is used as insert needs therefore to be phosphorylated. A Pfu product that is used as vector is ready to go since it is already unphsophorylated.
19. The construct will integrate at the effector locus corresponding to the effector fragment it contains and the effector::AvrRpt2$^{81\text{-}255}$ fusions will therefore be expressed from the native promoter of the effector.
20. If no TTSS-deficient derivative of the *P. syringae* strain used in your screen is available, you can sequence the full-length effector gene you found and clone it downstream of a constitutive promoter and fuse it to the AvrRpt2$^{81\text{-}255}$ reporter on a non-integrating vector. pBAV178 is a Gateway (Invitrogen) compatible vector developed for this purpose and can be requested from the authors. You can then mate this plasmid into any TTSS proficient and deficient *P. syringae* strain to test TTSS-dependent secretion.
21. A different algorithm for effector prediction is described in ***(15)***. The protocol described here can also be done in a different order, for example, looking first for genes downstream of conserved promoter elements and then looking for genes with amino acid biases.
22. In the case of *P. syringae*, also look for a high proline content instead of a high serine content in the first 50 amino acids and for an overall serine content of at least 10% over the whole protein. *P. syringae* effectors also often have cluster of serines or serines and prolines in the first 50 amino acids. *See also* Greenberg and Vinatzer ***(11)*** for more background information.
23. Shine-Dalgarno sequences are bacterial ribosome binding sites with the consensus AGGAGG located four to seven nucleotides up-stream of a gene's START codon.

24. However, there are mistakes in the databases and you should always be critical about any published annotation you find.
25. Sometimes transposase or insertion sequences are found between effectors and their *hrp* box increasing the distance between *hrp* box and effector. An effectcor may also be in an operon with non-effector encoding genes.
26. In case of *Ralstonia solanacearum* and *Xanthomonas* you will look for the PIP box or hrp_{II} promote element ***(13)***.

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4

In planta Expression of Oomycete and Fungal Genes

Thirumala-Devi Kanneganti, Edgar Huitema, and Sophien Kamoun

Summary

Large-scale genome sequencing projects have generated a wealth of sequence information for plant pathogenic microbes such as oomycetes and fungi. Functional genomic approaches are essential to exploit the sequence information to identify pathogen effector genes that trigger cellular and molecular responses in plant cells. This chapter describes two functional assays, agroinfiltration and agroinfection. These assays allow rapid functional expression of pathogen genes in plants and can be used in high-throughput screens.

Key Words: Transient gene expression; *Potato virus X*; functional genomics; oomycetes; fungi; effectors; *Nicotiana benthamiana*; *Agrobacterium tumefaciens*; agroinfiltration; agroinfection.

1. Introduction

Advances in sequencing technologies resulted in extensive collections of gene sequences from a plethora of eukaryotic plant pathogens, including oomycete and fungal species. One major thrust in this post genomics era is to identify genes that are important for pathogenesis and virulence. One class of such genes encodes so-called effectors that manipulate host cell structure and function either by facilitating infection (virulence factors) or by triggering defense responses (avirulence factors or elicitors). Typically, ectopic expression of single effector genes in plant cells leads to phenotypic effects. For example, expression of avirulence (*Avr*) genes in plant cells that contain the matching resistance (*R*) gene usually results in the hypersensitive response (HR ***[1]***). Also, expression of effector genes in susceptible hosts can lead to phenotypic responses that may reflect virulence function ***(2–6)***.

The use of plants for heterologous gene expression traditionally involved integration of a transgene into the plant genome ***(7)***. The main setback of this approach is the considerable time required for generating stable transgenic

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

plants. The time interval may vary from weeks to months depending on the plant species. Alternatively, ectopic gene expression also can be accomplished using such transient expression systems as *Agrobacterium tumefaciens*-based transient transformation (agroinfiltration), viral expression systems (agroinfection), and particle bombardment. Transient expression systems have a number of advantages over stable transformation. These assays are rapid and simple to perform. They can be applied to fully differentiated plant tissues, thus allowing the analysis of cell death-inducing genes without inducible promoters. Additionally, these assays are not influenced by chromosomal positional effects ***(8)***. Therefore transient expression assays have become popular in the study of plant–microbe interactions and also have been applied to high-throughput analyses ***(2,5,6,9,10)***.

This chapter describes two methods, agroinfiltration and binary *Potato virus X* (PVX) expression (PVX agroinfection), that have proved successful in our studies on effectors of *Phytophthora* ***(5,6,10)***. Despite some limitations, these assays are crucial to modern molecular plant pathology research. In addition, they meet the demand for efficient and robust high throughput functional analysis in plants.

1.1. Agroinfiltration

Agrobacterium tumefaciens is the most commonly used agent in plant transformation experiments. This bacterium is a ubiquitous pathogen of plants. It enters through natural wounds and causes tumors (crown galls) at infection sites. Translocation of transfer DNA (T-DNA) from a Ti plasmid (i.e., tumor-inducing plasmid) occurs after the virulence machinery of the bacterium is activated by low-molecular-weight phenolic compounds and monosaccharides that are released from wounded plant cells, combined with a slightly acidic environment ***(11)***. The agroinfiltration assay involves incubations of *A. tumefaciens* cell suspensions with 3'-5'-dimethoxy-4'-hydroxy acetophenone (acetosyringone). This phenolic compound mimics plant wounding, thereby inducing *vir* gene expression. This treatment is followed by the infiltration of cell suspensions into leaf panels, allowing transformation of accessible plant cells and leading to expression of the transgene(s) contained in the T-DNA region. Although chromosomal integration of T-DNA elements takes place during transformation, it is not known whether this is required for expression to occur. Nevertheless, the majority of plant cells in the infiltrated region express the transgene.

Ectopic expression of single pathogen genes in plant cells often leads to phenotypic effects. For instance, expression of bacterial, fungal, or oomycete *Avr* genes in plant cells that contain the matching *R* gene results in the HR ***(1)***.

In situations in which an expressed effector gene is not recognized, other phenotypic changes, such as chlorosis, cell enlargement, cell division, or necrosis, can be observed ***(4)***. In either case, phenotypic assessments of infiltrated leaf areas can help identify effector genes and aid in subsequent functional characterizations.

1.2. PVX Agroinfection

A number of plant viruses can be used as vehicles for transient gene expression in plants. RNA viruses can multiply to very high levels in infected plants, which makes them ideal vectors for gene expression. To engineer viral vectors, viral RNA genomes are reverse transcribed in vitro and cloned as full-length complementary DNAs in transcription vectors. Insertion of foreign genes into plant viral genomes can be achieved using the following methods:

1. Gene replacement, in which nonessential viral genes such as the coat protein gene are replaced by the gene of interest.
2. Gene insertion, in which the gene of interest is placed under the control of an additional strong subgenomic promoter.
3. Gene fusion, in which the gene of interest is translationally fused with a viral gene ***(8,12)***.

Among plant RNA viruses, PVX is widely used for expressing virulence and avirulence genes from viruses, bacteria, fungi, and oomycetes. The PVX genome was modified by incorporating a duplicated coat protein promoter sequence followed by a multiple cloning site for insertion of the gene of interest ***(13)***. Original constructs required in vitro transcription of PVX RNA followed by rubbing inoculation onto plant leaves. However, more recently, David Baulcombe and collaborators (Sainsbury Lab, Norwich, UK) developed binary PVX vectors in which the full-length PVX genome, flanked by the *Cauliflower mosaic virus* (i.e., CaMV) 35S promoter and the nopaline synthase terminator, was cloned in the T-DNA of an *A. tumefaciens* binary vector ***(14)***. Viral infection is initiated by wound inoculation of the recombinant *A. tumefaciens* strain onto leaves of host plants resulting in transfer of the T-DNA containing the PVX genome into plant cells. The PVX genome is then transcribed from the 35S promoter, resulting in virus particles that can move from one plant cell to another and spread systemically in the inoculated plants. Expression of the inserted gene is achieved during viral replication.

The PVX agroinfection assay has emerged as a robust and reliable system to identify virulence and *Avr* genes from microbial and viral pathogens. Expression screens using the PVX vector facilitated the isolation and study of *Avr* and effector genes from fungal, oomycete, bacterial, and viral plant pathogens ***(2,3,5,6,9,15–18)***.

2. Materials

2.1. Agroinfiltration

1. *Nicotiana benthamiana* seeds.
2. LB solid agar media plates supplemented with 50 µg of kanamycin and 25 mg of rifampicin/mL.
3. *A. tumefaciens* strain GV3101 (*see* **Note 1**).
4. *A. tumefaciens* strain GV3101 containing binary vector constructs (*see* **Notes 2–5**).
5. YEB medium: 5 g of beef extract, 1 g of yeast extract, 5 g of bacteriological peptone, 5 g of sucrose, and 2 mL of 1 *M* $MgSO_4$/L.
6. 3'-5' Dimethoxy-4'-hydroxy acetophenone (acetosyringone): 100 m*M* stock in dimethyl formamide or 70% ethanol.
7. 2-[*N*-Morpholino] ethane sulfonic acid (MES).
8. MMA infiltration medium: 5 g of MS salts, 1.95 g of MES, 20 g of sucrose, pH adjusted to 5.6 with 1 *M* NaOH, and 200 µ*M* acetosyringone/L (*see* **Note 6**).
9. 1-mL Syringe.

2.2. PVX Agroinfection

1. *N. benthamiana* seeds (*see* **Note 14**).
2. LB solid agar media plates supplemented with 50 µg kanamycin/mL.
3. *A. tumefaciens* strain GV3101 (*see* **Note 15**).
4. GV3101 harboring pGR106 or pGR106 carrying a reporter gene as a negative control.
5. GV3101 harboring pGR106-INF1 ***(17)*** as a positive control.
6. Sterile toothpicks.

3. Methods

3.1. Agroinfiltration

3.1.1. *Growing* N. benthamiana *Plants for Agroinfiltration*

1. Germinate *N. benthamiana* seeds in soil in a pot at 22 to 25°C with high light intensity. Cover the pots with cheesecloth to prevent drying and to provide adequate moisture.
2. After germination, remove cheesecloth and allow plants to grow for approx 1 to 2 wk.
3. Transplant 2-wk-old seedlings individually into separate Styrofoam cups containing soil and allow them to grow until they reach eight-leaf stage (*see* **Note 7**).

3.1.2. *Agroinfiltration Assay Procedure*

1. Streak recombinant *A. tumefaciens* strains onto LB solid agar media plates supplemented with 50 µg kanamycin and 25 µg rifampicin/mL and incubate at 28°C for 2 to 3 d.
2. Inoculate 3 mL of YEB cultures containing 50 µg of kanamycin and 25 µg of rifampicin/mL, with the recombinant *A. tumefaciens* strains and grow overnight (28°C, approx 225 rmp).

3. Inoculate large YEB media suspensions (25–300 mL; *see* **Note 8**), containing 50 μg of kanamycin, 25 μg rifampicin/mL, and 2 μ*M* acetosyringone with the overnight culture. Grow cultures overnight at 28°C to an OD600 of approx 1.
4. Harvest the cells by centrifugation (4000*g* for 10 min), pour off the supernatant and resuspend the pellet in MMA medium to an OD of 2 (*see* **Note 9**).
5. Incubate and shake cells at room temperature for 1 to 3 h.
6. Place *A. tumefaciens* suspensions into a syringe. Carefully invert the leaf and hold the lower side up. Support the infiltration site with your index finger and place the syringe against the leaf and index finger. While applying gentle pressure to the leaf, inject the suspension slowly from the syringe. Successful infiltration can be seen as the *Agrobacterium* suspension spreads from the infiltration site into the leaf (*see* **Note 10**). A movie on "how to agroinfiltrate" is availableat (http://www.sainsbury-laboratory.ac.uk/david-baulcombe/Services/AgroInfiltrationHP.htm).
7. Incubate the plants in a growth chamber or confined space at 22°C (*see* **Note 11**).
8. Response should be visible in 2 to 3 d after infiltration (*see* **Notes 12** and **13**).

3.2. PVX Agroinfection

3.2.1. Growing N. benthamiana Plants for Agroinfection

1. Germinate *N. benthamiana* seeds in soil in a pot at 22 to 25°C with high light intensity. Cover the pots with cheesecloth to prevent drying and to provide adequate moisture.
2. After germination, remove cheesecloth and allow plants to grow for approx 1 to 2 wk.
3. Transplant 2-wk-old seedlings individually into separate Styrofoam cups containing soil.
4. Allow plants to grow until they reach four-leaf stage (*see* **Note 16**).

3.2.2. Agroinfection Assay Procedure

1. Streak recombinant *A. tumefaciens* strains onto LB solid agar media plates supplemented with 50 mg of kanamycin/mL and incubate at 28°C for 2 to 3 d (*see* **Notes 17** and **18**).
2. Toothpick-inoculate individual clones on the lower leaves of *N. benthamiana* plants by dipping a wooden sterile toothpick in a culture of the recombinant *A. tumefaciens* strain and piercing the leaves on both sides of the mid vein (*see* **Notes 19–21**).
3. Incubate the plants in a growth chamber or confined space at 22°C (*see* **Note 22**).
4. Response should be visible starting from 7 d after inoculation (*see* **Note 23**).
5. Strains carrying the recombinant constructs should be examined for altered viral symptoms and compared with the control strains. Strains carrying the vector pGR106 induce systemic mosaic symptoms, and strains carrying pGR106-INF1 induce local HR lesions ***(5)***.

4. Notes

4.1. Agroinfiltration

1. We prefer to use GV3101 because it electroporates at high frequency (>10^8 cfu/μg of DNA). This streamlines the cloning procedure, as ligation mixtures can be directly electroporated into *Agrobacterium*.
2. Several binary vectors can be used. Vectors based on the pCB300 series ***(19)*** or the pAvr9 vector ***(1)*** allow high expression of the candidate gene and have worked well in our hands.
3. Agroinfiltration can be used with large (>2 kb) genes. PVX does not permit expression of genes exceeding 2 kb.
4. It is critical to include proper controls for each experiment. An *A. tumefaciens* strain containing a vector without gene insert is recommended as a negative control. Binary vectors containing genes expressing marker proteins, such as β-glucuronidase (i.e., GUS) or green fluorescent protein, can be used to verify the level of transformation by agroinfiltration.
5. Several transgenes can be delivered into the same cell with agroinfiltration system facilitating simultaneous expression of interacting proteins (i.e., Avr and R proteins) or assembly of multimeric proteins.
6. It is recommended to make fresh MMA media by adding acetosyringone just before washing and incubation of the cell suspensions.
7. *N. benthamiana* plants that have healthy and fully developed leaves are desired in this assay. Infiltration of senescing leaves can lead to necrosis and reduced transformation rates.
8. The amount of infiltration media to be used depends on the size of the experiment and infiltration efficiency.
9. Infiltration with dense *A. tumefaciens* suspensions can lead to background necrosis. These problems can be avoided by using suspensions with lower OD600 values.
10. It is strongly recommended to practice and refine one's infiltration technique with water. Some users prefer to cause a slight wound on the leaf using a needle or a razor blade at the site of injection, which will facilitate infiltration of the bacterial solution.
11. Incubation temperatures of infiltrated plants should not exceed 28°C. *A. tumefaciens* transformation efficiency and transgene expression peaks at 22°C ***(20)***.
12. Detectable transgene expression should occur 2 to 3 d after infiltration. However, the timing of phenotypic changes varies depending on the effector tested.
13. The agroinfiltration system, unlike viral vectors, does not permit systemic expression of the foreign gene.

4.2. PVX Agroinfection

14. PVX agroinfection is limited to host plants, such as *N. benthamiana*, *Nicotiana tabacum*, *Lycopersicon esculentum*, and *Solanum tuberosum*.

15. We use GV3101 because it electroporates at high frequency (>10^8 cfu/μg of DNA). This streamlines the cloning procedure since ligation mixtures can be directly electroporated into *Agrobacterium*.
16. Younger plants at three to four leaf stages are preferable for inoculation if systemic symptoms are sought. For local responses, multiple clones can be inoculated on a single leaf. *See also* the method described by Takken et al. ***(9)*** for inoculation of 96 clones in tobacco leaves.
17. Always use pGR106 empty vector or a pGR106 carrying a reporter gene, such as *gfp*, as a negative control. The presence of an insert slows down virus infection so the use of a control vector carrying an insert of the same size as the candidate gene might be more appropriate than the empty vector.
18. It is advisable to use fresh cultures that are not older than 4 d.
19. Excess amount of *A. tumefaciens* can be used for toothpick inoculation.
20. Three leaves per plant can be used to serve as triplicates.
21. Inoculate a minimum of four plants for each construct.
22. Incubation temperatures of infected plants should not exceed 28°C. *A. tumefaciens* transformation efficiency peaks at 22°C. This is also the optimal temperature for virus replication.
23. Symptoms should be scored every day from 7 d postinoculation and until 15 to 21 d after inoculation.

Acknowledgments

We thank past and present members of the laboratory for helping to refine the described protocols, J. Kapila for providing us with the original agroinfiltration protocol, and I. Malcuit and D. Baulcombe for providing pGR106 and help with the PVX system. Research on functional genomics of *Phytophthora*-plant interactions is supported by NSF Plant Genome Research Program grant DBI-0211659. Salaries and research support were provided, in part, by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

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5

Use of Nipponbare BAC Clones for Physical Mapping of an *R* Gene Locus in Rice

Jong-Seong Jeon and Pamela C. Ronald

Summary

Major advances in rice genomics during the last few years have made positional cloning in rice much more efficient. Nipponbare is a model rice genotype being sequenced by the International Rice Genome Sequencing Project Consortium. Here, we describe an efficient procedure of the construction of physical map for positional cloning of resistance gene (*R*) using the Nipponbare genetic resources. This advanced strategy should be useful for the efficient identification of agronomic important *R* genes from many resistant rice genotypes, including wild rice species.

Key Words: Rice; *R* gene; Nipponbare; BIBAC library; saturation mapping; positional cloning.

1. Introduction

Flor ***(1)*** proposed a model based upon the genetic studies using flax and the flax rust pathogen. The "gene-for-gene" model predicts that plant resistance will occur only when a plant possesses a dominant resistance gene (*R*) and the pathogen expresses the complementary dominant avirulence gene (*Avr*). An alteration or loss of the plant resistance gene or the pathogen *Avr* determinant leads to disease on the host as the outcome. The *R* gene products are hypothesized to act as receptors for the products of the avrulence locus. The model holds true for many host–pathogen interactions.

Cloning and characterization of several disease resistance genes in different plant species has revealed common structural features in their predicted protein products, including nucleotide binding site (NBS) and leucine-rich-repeat (LRR) domains, suggesting that common mechanisms for perception and transduction of pathogen signals exist in diverse plant species ***(2)***. In rice, the most serious fungal disease of rice is blast caused by the fungus *Magnaporthe grisea*

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

and the most serious bacterial diseases of rice in Africa and Asia is bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Of the four rice disease resistance genes cloned from rice so far, three, *Xa1* ***(3)***, *Pib* ***(4)***, and *Pita* ***(5)***, possess sequences encoding the NBS–LRR domains, whereas one, *Xa21*, codes for an LRR receptor kinase-like protein ***(6)***.

The *R* gene-mediated resistance is known to be an economical method to control losses in the field. The combined presence of *R* loci ensures a mechanism for conferring long-term and durable resistance ***(7)***. Using molecular markers of isolated genes or tightly linked to resistance loci, simultaneous selection of multiple resistance loci, called marker assisted selection, has been facilitated for pyramiding *R* genes in a certain cultivar.

Positional cloning, also called map-based cloning, is the process of identifying the genetic basis of a phenotype, i.e., resistance, by looking for linkage to markers whose physical location in the genome is known. The amount of effort required for map-based cloning of genes in rice has dropped dramatically in recent years. Saturation mapping, a new approach to produce markers in a very small interval of several hundred kilobase pairs, is indispensable for positional cloning.

During the last few years major advances in rice genomics have made positional cloning in rice much more efficient. A high-density genetic linkage map and a YAC-, PAC-, and BAC-based contig map have been constructed for the rice cultivar Nipponbare ***(8)***. More than 110,000 sequence-tagged connectors have been generated by sequencing both ends of every BAC clone ***(9)***. A fingerprint-based contig of BAC clones has been anchored with restriction fragment-length polymorphism markers onto the genetic map ***(10)***. In addition, Nipponbare is the rice genotype being sequenced by the International Rice Genome Sequencing Project Consortium ***(11)***.

Nipponbare appeared to be susceptible to many *M. grisea* strains. Recent studies, however, demonstrated that the genetic resource of Nipponbare lacking an *R* gene can be efficiently used to develop markers required for saturation mapping of the *R* locus ***(12–14)***. Here, we describe in detail an efficient method of the construction of physical map for positional cloning in rice using the Nipponbare genetic resources. First, initial markers linked to an *R* gene are mapped on the high-density genetic map of Nipponbare/Kasalath. Secondly, Nipponbare BAC clones physically spanning the region are identified using the Arizona Genomics Institute (AGI) database (http://www.genome.arizona.edu/fpc/rice/). Third, additional genetic markers for saturation mapping are produced using subclones of the identified BACs. Fourth, a small interval of Nipponbare corresponding to the *R* gene region is delimited by determining recombination breakpoints. Fifth, a physical map of the R locus is constructed using flanking markers and a BIBAC library generated from the *R* gene-containing line.

2. Materials

2.1. Nipponbare BAC DNA Isolation

1. Terrific broth (TB) liquid medium (1.0 L): To 900 mL of double-distilled H_2O (ddH_2O), add 12 g of bacto-tryptone, 24 g of bacto-yeast extract, and 4 mL of glycerol. After sterilizing by autoclaving, allow the solution to cool to less than 60°C, and then add 100 mL of a sterile solution of 0.17 *M* KH_2PO_4 and 0.72 *M* K_2HPO_4.
2. ddH_2O sterile water
3. Chloramphenicol
4. Resuspension buffer: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* ethyelene diamine tetraacetic acid (EDTA), 100 μg/mL RNaseA.
5. Lysis buffer: 200 m*M* NaOH, 1% sodium dodecyl sulfate.
6. Neutralization buffer: 3 *M* potassium acetate, pH 5.5.
7. Miracloth.
8. Isopropanol.
9. 10 m*M* Tris-HCl, pH 8.0.
10. RNase.
11. Phenol:chloroform (1v:1v).
12. Absolute ethanol.
13. 70% Ethanol.
14. Agarose.

2.2. Sublibrary Construction of BAC DNA

1. *Sau*3AI.
2. QIAquick Gel Extraction Kit, Qiagen.
3. pBluescriptII SK(+) (Stratagene).
4. ElectroMAX DB10B cells (Life technologies).
5. CELL-PORATOR (Gibco BRL).
6. Ampicillin.
7. LB medium (per 1 L): 10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl.
8. SOC medium (1.0 L): Mix 25 g of LB broth, 10 mL of 1.0 *M* KCl stock solution, and 970 mL of ddH_2O. Adjust pH to 7.0 and autoclave. Immediately before use, add 10 mL of 1.0 *M* $MgSO_4$ solution and 10 mL of 2.0 *M* glucose solution.
9. 50X TAE (1.0 L): Mix 242.2 g of Tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 *M* EDTA (pH 8.0) stock solution, and add ddH_2O to 1 L.

2.3. BIBAC Library Construction

1. *Hin*dIII.
2. 0.5 *M* EDTA (pH 8.0) solution.
3. Low-melting-point agarose (BMA, SeaPlaque GTG Agarose).
4. CHEF DR II system (Bio-Rad).
5. Low-range PFG marker (NEB).

6. β-Agarase I (1.0 U/μL; NEB).
7. Nitrocellulose filters (Millipore VSWP02500).
8. 10% (w/v) polyethylene glycol (PEG).
9. pBIGRZ vector: Request to Dr Shinji Kawasaki.
10. T4 ligase with 10X buffer (NEB).
11. X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactoside).
12. Isopropylthiogalactoside (IPTG).
13. Kanamycin.
14. Freezing media: 2.5% w/v LB broth, 13 m*M* KH_2PO_4, 36 m*M* K_2HPO_4, 1.7 m*M* sodium citrate, 6.8 m*M* $(NH_4)_2SO_4$, and 4.4% v/v glycerol. Autoclave and allow media to cool to less than 50°C. In a laminar-flow hood, add $MgSO_4$ stock solution to a final concentration of 0.4 m*M*. Immediately before use, add antibiotics solution to each liter of freezing media.

3. Methods

3.1. Identification of Nipponbare BAC Clones Encompassing Initial Markers

1. To construct the high-resolution map of an *R* gene, initial co-segregating markers linked to the *R* gene should be identified by the bulked segregant analysis combined using randomly amplified polymorphic DNA ***(15)*** and/or amplified fragment-length polymorphism methods ***(14)*** using a small population of 50 individuals.
2. To pinpoint the Nipponbare genomic region corresponding to the R locus, sequence the identified initial co-segregating markers, open the AGI WebBSS URL (http://www.genome.arizona.edu/fpc/rice/), enter the query sequences, and blast your sequence against Nipponbare sequenced clones (*see* **Note 1**).
3. If the **step 2** procedure fails to identify a positive hit(s), carry out a colony hybridization experiment using your markers as a probe with two (*Hin*dIII and *Eco*RI) BAC libraries of Nipponbare that are provided by the Clemson University Genome Institute (CUGI; http://www.genome.clemson.edu/). This experiment may allow you to identify a large physical region consisting of Nipponbare BAC clones encompassing the markers (*see* **Note 2**).
4. Go to the WebFPC (http://www.genome.arizona.edu/fpc/rice/WebAGCoL/WebFPC/) and click the contig including BAC clone(s) matching your sequences.
5. Choose 6 to 20 (depending on the distance of the initials markers) of the contiguous Nipponbare BAC clones to develop internal markers that flank your gene. Details of the contig with respect to total number of BAC clones are accessible at http://www.genome.arizona.edu/fpc/rice/ (*see* **Notes 3** and **4**).
6. The Nipponbare genomic resources are provided upon request.

3.2. Development of Markers for Use in Saturation Mapping

3.2.1. Isolation of BAC DNA

1. Inoculate the *E. coli* strain (Nipponbare BAC clone) into 100 mL of TB liquid medium supplemented with antibiotics chloramphenicol (12.5 mg/L) and grow at 37°C for 12 to 16 h with vigorous shaking.

2. Harvest the bacterial cells in a blue cap tube by centrifugation at 1500*g* for 10 min.
3. Suspend the bacterial pellet in 10 mL of resuspension buffer.
4. Add 10 mL of lysis buffer, mix gently but thoroughly by inverting several times, and incubate at room temperature for 5 min.
5. Add 10 mL of neutralization buffer, mix immediately but gently by inverting several times, and incubate on ice for 15 min.
6. Centrifuge the reaction at 1500*g* at room temperature for 20 min.
7. Transfer the supernatant solution (approx 25 mL) containing plasmid DNA into new tube by filtering over miracloth promptly.
8. Add two-thirds volume (approx 17 mL) of isopropylalcohol and shake the tubes gently.
9. Centrifuge at 1500*g* at room temperature for 15 min.
10. Allow the pellets to dry at room temperature.
11. Dissolve 500 µL of 10 m*M* Tris-HCl (pH 8.0) with RNase (20 µg/mL) and transfer the solution into an Eppendorf tube.
12. Add 500 µL of phenol:chloroform (1 v:1 v) and mix gently.
13. Centrifuge the mixtures at full speed for 5 min and transfer the supernatant (approx 450 µL) into a new tube.
14. Add 800 µL of absolute ethanol, mix well, and centrifuge at full speed for 5 min.
15. Wash DNA with 500 µL of 70% (v/v) ethanol.
16. Dry the pellets and resuspend in 100 µL of 10 m*M* Tris-HCl (pH 8.0).

3.2.2. Sublibrary Construction of BAC DNA

1. Perform a series of partial restriction digests of each BAC clone with *Sau*3AI by using different amount of enzyme and the digestion time. Once the optimal conditions for producing fragments of 0.2 and 2.0 kb are determined, perform a mass digestion using several clones.
2. Separate the digestion onto 0.8% agarose gel containing 1X TAE buffer.
3. Cut the 0.2- to 2.0-kb fragments and elute DNA fragments using QIAquick Gel Extraction Kit (Qiagen), a commercial gel extraction kit.
4. Ligate the fragments with a *Bam*HI-digested and dephosphorylated pBluescriptII SK(+) or a suitable cloning vector for approx 4 to 12 h.
5. Transform the ligates into ElectroMAX DB10B cells (Life Technologies) by using CELL-PORATOR (Gibco BRL) with wet ice.
6. Remove cells from microelectroporation chamber and immediately add to 1.0 mL of SOC medium.
7. Shake gently (37°C) for 1 h.
8. Spread 10 to 100 µL on LB plates with 100 µg/mL ampicillin.
9. Incubate overnight at 37°C.
10. Randomly pick up 150 recombinant white clones, culture in LB liquid media with 100 µg/mL ampicillin, and isolate plasmids from the overnight cultured cells by a minipreparation procedure ***(16)***.

3.2.3. Development of Polymorphic Markers

1. Amplify cloned inserts by PCR using T3 and T7 primers under the following incubation condition: 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

2. With several restriction endonucleases, for examples, *Eco*RI, *Bam*HI, *Hin*dIII, or etc., digest genomic DNAs isolated from resistant and susceptible parents used in the generation of mapping population.
3. Separate the digestion on 0.8% agarose gel, and transfer DNAs onto a nylon membrane according to Sambrook et al. ***(16)***.
4. Perform DNA gel-blot hybridization using the PCR products as probes and the digested DNAs as targets according to Sambrook et al. ***(16)***.
5. Identify polymorphic markers which show a clear polymorphism between the resistance donor and the susceptible recipient genotypes at the DNA gel-blot analysis (*see* **Note 5**).
6. Determine the sequences of the newly generated markers by using T3 and T7 primers (*see* **Note 6**).
7. To use the markers most efficiently in the analysis of a large population, they should be converted to PCR-based markers. Design PCR primers from the sequence information of new markers using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).
8. To develop the PCR markers, amplify DNA fragments of both parents using marker-specific PCR primers at an appropriate annealing temperature, usually between 56 and 58°C.
9. Determine directly the sequence of both fragments by using PCR primers.
10. Select first the sequences containing a different restriction enzyme digestion pattern between parental genotypes to develop cleaved amplified polymorphic sequence (CAPS) markers (*see* **Note 7**).
11. To confirm that the newly developed CAPS markers show a polymorphic band patterns, digest about 10 µL (approx 200 ng PCR product) with appropriate restriction enzymes for an hour at 37°C, separate the digestions on approx 1.0% to 2.0% agarose gel, and examine the digested band patterns.

3.3. Saturation Mapping Using Newly Developed Markers

1. Isolate genomic DNA from leaves (approx 100 mg) of all growing plants according to Chen and Ronald ***(17)***. At a final step, dissolve the final DNA products in 100 µL of 10 m*M* Tris-HCl (pH 8.0; *see* **Note 8**).
2. Perform PCRs using marker-specific primers in a 30-µL reaction using 1 µL (approx 50 ng) of genomic DNAs.
3. Digest each 5 µL of the PCRs in a 30-µL reaction for 1 h with selected restriction enzyme producing DNA polymorphism between both parents, and separate a half volume of the digestion on an approx 1.0 to 2.0% agarose gel (*see* **Note 9**).
4. Determine genotypes of 50 susceptible individuals at loci of all developed PCR markers by repeating **steps 2** and **3** (*see* **Note 10**).
5. Construct a genetic linkage map of the region containing your gene using Mapmarker software ***(18)***. Use all segregation dataset generated from the analysis including the initially established cosegregating markers' data set.
6. Present map distances in cM between markers according to Kosambi function ***(19)***.
7. A prescreening strategy to identify plants with rare recombination events around the *R* gene is useful using the closest flanking markers (*see* **Note 11**). Without the

analysis of phenotypes of all plants, this experiment can identify more recombinants between both markers. Enlarge the mapping population to approx 2000 individuals until a small physical *R* gene region of less than 200 kb is narrowed down by repeating **steps 1** to **6** (*see* **Note 12**).

8. Confirm phenotypes of all identified individuals displaying the rare recombination events in the progeny from each line by infecting rice pathogen, for example, *Magnaporthe grisea* or *Xoo*. This will distinguish heterozygous from homozygous for the resistance donor genome, enabling one to constructing an accurate map.
9. Construct a final high-resolution map of the *R* gene using all dataset by repeating **steps 5** and **6**.

3.4. Construction of BIBAC Contig Spanning the R Gene Locus

3.4.1. BIBAC Library Construction

1. Isolate high-molecular-weight DNA from young leaves of 3-wk-old rice plants carrying the *R* gene by the CTAB method described in Murray and Thompson (***[20]*** *see* **Note 13**).
2. Digest high-molecular-weight DNA partially with *Hin*dIII in a 50-μL reaction. To determine the conditions that yield a maximum percentage of fragments between 25 and 40 kb, perform a series of partial restriction digests by using different amount of enzyme and the digestion time
3. Add 5 μL of 0.5 *M* EDTA to stop the reaction.
4. Load into a 0.8% low-melting agarose gel and separate in 1X TAE buffer by a pulsed-field gel electrophoresis (PFGE) device (CHEF DR II system, Bio-Rad) at 160 V using a 6-s initial and 6-s final switch time for 16 h at 14°C.
5. Cut the purified DNA from the low-melting-point agarose slice containing 25 to 40 kb (*see* **Note 14**).
6. Record the weight of the agarose slice on a balance and transfer the slice to a sterile Eppendorf tube.
7. Incubate in 2 vol of 1X β-Agarase I buffer on ice. Repeat this process five times. This step removes electrophoresis buffer from the gel slices which possibly could interfere with ligation.
8. Decant the final wash and place the tubes in a 70°C water bath for 5 min or until all of the agarose has become liquid.
9. Quickly transfer the tubes to a 42°C water bath and add β-Agarase I to each tube so that there is 1 U of enzyme for every 200 μL of molten low-melting-point agarose (*see* **Note 15**).
10. Gently swirl the contents of each tube and incubate the tubes at 42°C for an hour.
11. To concentrate the DNA solution, perform dialysis for approx 3 h against 30 mL of 10% PEG solution in a 90-mm Petri dish using a particular nitrocellulose filter (Milipore; cat. no. VSWP02500 filter, pore size 0.025 μm).
12. Place insert DNA (approx 50 ng), the *Hin*dIII-digested and dephosphorylated pBIGRZ vector (approx 10 ng), and T4 DNA ligase (10 U) with buffer in 0.5-mL microcentrifuge tubes (*see* **Note 16**). Gently mix and do not vortex as this may shear the insert DNA. The pBIGRZ vector ***(21)*** is available from Dr. Shinji Kawasaki (*see* **Note 17**).

13. Incubate the ligation reactions at 16°C overnight.
14. Place ligation reaction tubes in a 65°C water bath for 20 min to heat-denature the enzyme.
15. To desalt the ligated DNA, place the ligation reaction on the Milipore VSWP filter as described in **step 11**.
16. Using pipet tips, transfer all of the desalted ligation reactions into a single 0.5-mL microcentrifuge tube.
17. Transform the ligation mix by electroporation using a Cell-Porator (Gibco-BRL) into ElectroMAX DB10B cells (Life Technologies).
18. Remove cells from micro-electroporation chamber and immediately add to 1.0 mL of SOC medium.
19. Shake gently (37°C) for 1 h.
20. Spread approx 10 to 100 μL on LB plates with 100 μg/mL Kanamycin, 240 mg/ L X-gal, and 80 mg/L IPTG (*see* **Note 18**) so that approx 500 clones grow in a Petri dish.
21. Incubate overnight at 37°C.
22. Pour 4 mL of the freezing media per Petri dish and collect all colonies (approx 500) to form a BIBAC pool (*see* **Note 19**). Keep the pool at –70°C.
23. Repeat **steps 18–23** until 200 BIBAC pools are ready (*see* **Note 20**).

3.4.2. Screening of BIBAC Library for Construction of BIBAC Physical Contig

1. Fifty-microliter stock solution of each 200 BIBAC pools are cultured in 100 mL of kanamycin-containing TB media for overnight, and isolate total BIBAC plasmids as described in **Subheading 3.2.1.**
2. A pooling system for a PCR-based procedure is prepared as follows: equivalent amounts (10-μL aliquot) of DNAs purified from 10 pools are mixed to make a super pool, producing 20 super pools (*see* **Note 20**).
3. Perform a first round of PCR using each 10 ng of DNA of 20 super pools and marker-specific primers in a 30-μL reaction (*see* **Note 21**).
4. Separate a part (10 μL) of the PCR on 1.0% agarose gel and identify a super pool(s) showing a positive hit.
5. In a second round of PCR, screen the individual 10 pools making up the super pool using the same PCR condition by repeating **steps 3–5**.
6. Finally, screen more than 2000 individual clones of the identified pool by a colony blot hybridization Sambrook et al. ***(16)*** using the PCR products amplified in the second PCR as probes.
7. Culture the isolated clone in Kanamycin-containing TB media and purify BIBAC plasmid DNA as described in **Subheading 3.2.1.**
8. Determine both BIBAC end sequences using T3 and T7 primers.
9. Develop the BIBAC end sequence-specific primers for use subsequently in chromosome walking (*see* **Notes 22** and **23**).
10. Repeat **steps 3–10** until the genomic region of the *R* gene is completely covered by BIBAC clones (*see* **Note 24**).

4. Notes

1. AGI sequenced the ends of Nipponbare BAC library (92,160 clones) as part of an international effort to sequence the rice genome.
2. The filters from a *Hin*dIII BAC (OSJNBa) library of Nipponbare consist of 36,864 clones/set with an average insert size of 130 kb and corresponding to 10 genome equivalents. An *Eco*RI BAC (OSJNBb) library has an average insert size of 120 kb and 55,296 clones.
3. Most contigs consist of more than 100 BAC clones and at least 30 developed markers.
4. Nipponbare BAC contigs should provide new markers located on the Nipponbare/Kasalath linkage map (http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html), including 3267 markers. In the other way to identify Nipponbare genomic resources encompassing your markers, open the INtegrated rice genome Explorer (http://rgp.dna.affrc.go.jp/giot/INE.html), which is a database integrating the genetic map, physical map and sequencing information of rice genome, and look for BAC/PAC/YAC clones with markers.
5. The clones that are monomorphic between the parents, those with comparatively weak hybridization signals or those with multiple bands are considered inappropriate for further analysis.
6. The clones encoding a "nucleotide binding site plus leucine-rich repeat" (NBS–LRR) motif are included first. Select polymorphic markers evenly distributed or homologous to known resistance genes.
7. To avoid an error caused during PCR amplification, sequence a mixture of PCR product, instead each PCR product cloned into T-vector. In case some markers cannot be converted to CAPS markers, the amplified corresponding regions of both markers from parents are directly compared the sequence of the PCR products. This can identify a few nucleotide differences between parents.
8. The rapid DNA minipreparation method developed by Chen and Ronald ***(17)*** is suitable for avoiding cross-contamination and for PCR applications.
9. Using a higher percentage of agarose gel helps produce a clear difference between relatively small sizes of digested DNAs.
10. Fifty susceptible individuals are suitable for construction of an initial fine map. Because the score of susceptibility is more reliable than resistance because of escapes from the inoculum, first analyze all susceptible plants to make an accurate map. If skewed recombination events are expected at the locus, use of a second mapping population can be a better way.
11. When limited quarantine facilities are used for blast inoculations, the prescreening strategy is useful.
12. The recombinant lines are further analyzed using internal markers that are additionally developed later.
13. Nipponbare does not carry the *R* gene, therefore construction of a BIBAC library from the resistance donor cultivar is necessary.
14. Use the low-range PFG marker (NEB) as size standards. The marker lanes are cut and stained with ethidium bromide. Make small incisions in the marker gel slices

at 25 to 40 kb on a UV light box. Reconstruct the gel by placing the unstained gel beside the marker gel slice, and cut the 25- to 40-kb DNA-containing gel block. Never expose the genomic DNA to UV light as this will cause a break of size-selected genomic DNA.

15. β-Agarase I (NEB) works best on gels made with Tris-acetate buffer. For gels made with Tris-borate buffer, doubling the required amount of β-Agarase I is recommended.
16. If a 5:1 ratio of insert to vector does not produce a satisfactory outcome, change the ratio of insert to vector to improve the results. To use a high quality of BIGRZ vector, purify the vector by the CsCl-ethidium bromide gradient centrifugation method ***(16)***. Heat-killing HK Thermolabile phosphatase (Epicentre Technologies) easily and completely is useful. Avoid a damage of the vector DNA during dephosphorylation to prevent an appearance of false-positive clones.
17. The BIGRZ vector is capable of about 30 kb average insert. It usually yields a high transformation efficiency of rice. A BIBAC library consisting of relatively smaller insert sizes may be sufficient or even preferable for certain applications, for examples, constructing a small contiguous genomic region and complementing the clones into wild-type (i.e, susceptible) rice plants.
18. Use of a higher amount of X-gal facilitates to distinguish blue colonies from white ones.
19. After incubating test plates overnight, determine the titer of the transformation reaction and the percentages of white and blue colonies. If more than 80% of the colonies are white, select randomly 20 white colonies and perform a *Hin*dIII digestion to determine an average size of inserts. A high percentage (more than 40%) of blue clones indicates possible problems during vector preparation, ligation, or transformation.
20. In rice, the library should carry more than 100,000 clones with an average DNA insert size of 30 kb, a 5X genome equivalent. This strategy is efficient for screening a large library with small sizes of inserts. The alternative strategy of picking more than 100,000 individual clones is time-consuming and laborious.
21. To span the physical region containing the *R* gene, use four markers for the first library screening.
22. The sequenced information of each end of the isolated BIBAC clones is used to generate PCR products to screen the library again to extend the physical region. By repeating this approach, identify additional clones spanning the region.
23. In case some BIBAC-end sequences are homologous to transposable elements, they cannot be used as probes for BIBAC library screening due to their repetitive nature. To fill out the interval, subclones isolated from the *Sau3*AI shotgun library of the BIBAC clone are utilized to find the linking clone as described in **Subheadings 3.2.2.** and **3.2.3.**
24. A small missing region can be simply amplified and confirmed by its sequence analysis.

Acknowledgments

The methods described here were developed with supports from a United States of Department of Agriculture grant (no. 2003-35301-13245), Crop Functional Genomics Center grant, Korea, and Plant Metabolism Research Center grant, Kyung Hee University, Korea.

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6

Agrobacterium-Mediated Transformation to Create an Insertion Library in *Magnaporthe grisea*

Sara L. Tucker and Marc J. Orbach

Summary

Magnaporthe grisea is the causal agent of rice blast disease and represents a model organism for the study of fungal plant–pathogen interactions. Pathogenicity is a complex phenotype, which is carefully orchestrated by the fungus and begins with recognition and infection of the host plant, followed by growth within the plant cells, and finally dissemination to the next host and continuation of the fungal life cycle. Certain genes must condition the ability of a pathogenic fungus to infect and cause disease symptoms. To learn more about the infection process and the genes that are involved in the complex interplay between *M. grisea* and rice, we used an insertional mutagenesis approach to attempt to randomly disrupt all genes in the fungal genome. Two transformation approaches were used to build a library of insertion strains in *M. grisea*. Polyethylene glycol/$CaCl_2$-mediated protoplast transformation was the initial method we used and resulted in the generation of just more than 17,000 insertion strain lines. Later *Agrobacterium tumefaciens*-mediated transformation was adopted and the final number of insertional mutant strains of *M. grisea* strain 70-15 generated was more than 57,000. Here, we describe the methods used for *A. tumefaciens*-mediated transformation of *M. grisea* and the optimized protocols we have developed to enable high-throughput fungal transformation. Further details of this optimization can be found elsewhere.

Key Words: *Magnaporthe grisea*; *Agrobacterium tumefaciens*-mediated transformation; rice; insertional mutagenesis; pathogenicity; appressorium.

1. Introduction

This chapter describes the methods used for *Agrobacterium tumefaciens*-mediated transformation of *Magnaporthe grisea* and the optimized protocols we have developed to enable high-throughput fungal transformation. Further details of this optimization can be found elsewhere ***(1)***.

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

A. tumefaciens is a plant pathogen that is naturally capable of causing tumors by transferring DNA to approx 90 families of dicotyledonous plants, causing disease in 600 species (for reviews, *see* **refs**. ***2–4***).

The interaction between *Agrobacterium* and its plant host involves a complex series of chemical signals. These signals include neutral and acidic sugars, phenolic compounds, opines (crown gall-specific molecules synthesized by transformed plants), Vir (virulence) proteins, and the transfer DNA (T-DNA) that is ultimately passed from the bacterium to the plant cell ***(3)***. The T-DNA transfer process initiates after *A. tumefaciens* perceives phenolic and sugar compounds that are released from wounded plant cells. These compounds, including the phenolic chemical acetosyringone, act as inducers of the bacterial *vir* genes and are detected through the VirA sensory protein ***(3)***. Autophosphorylation of VirA protein and the subsequent transphosphorylation of VirG protein result in the activation of *vir* gene transcription. During tumor induction, *Agrobacterium* transfers part of its Ti- (tumor-inducing) plasmid, the T-DNA, which is flanked by 24-bp imperfect direct repeats, to plant cells. The T-DNA then randomly integrates into the plant nuclear genome. Molecular biologists have taken advantage of the unique biology of *Agrobacterium* and manipulated the T-DNA region by removing genes for the biosynthesis of plant hormones and opines, replacing them with various selectable markers, reporter genes or specific gene disruption constructs. To date, at least 22 fungal species representing 16 different genera of fungi have been successfully transformed using *A. tumefaciens*. Here, we describe *Agrobacterium*-mediated transformation of the rice blast fungus, *Magnaporthe grisea*. Two *Agrobacterium* strains were used to generate an insertion strain library in *M. grisea* strain 70-15 ***(5)***. Strain AGL1 ***(6)*** contains the pCAMBIA1300-based binary vector pBHt-2 ***(7)***. Strain EHA105 ***(8)*** contains the binary vector pAD1624 ***(9)*** and is called AD973. Both binary vectors contain the hygromycin B resistance gene from pCB1004 ***(10)*** between the T-DNA borders. The two strains differ in induction of the vir genes with AD973 containing a constitutive *vir*G gene (*virG*N54D) on the plasmid pAD1624, which facilitates transformation without the need to add the *vir* gene inducer acetosyringone ***(11)***.

After generation of the transformed *M. grisea* insertion lines, various phenotypic assays were performed to allow characterization of the strains that had been generated. All assays were optimized to be carried out in a high-throughput manner, which was essential to all aspects of this project. The assays enabled us to screen for alterations in pigmentation, growth rate, conidiation, auxotrophy, and pathogenicity compared with wild-type strain 70-15. To increase the range of phenotypes assayed and also the number of pathogenicity mutants identified, a further test to determine appressorium formation using inductive glass mirror was included. This assay led to the identification of *M. grisea*

insertion line strains that showed various phenotypes, such as abnormal spore morphology or germ tube formation, failure to differentiate an appressorium or multiple appressoria, or had misshapen appressoria. Of 11,326 strains screened for appressorium defects by this in vitro assay, more than 1% (122) had visible defects. Ninety-nine of these strains were screened on two rice cultivars (M-202 and Maratelli) for defects in pathogenicity. Of these, 71 strains exhibited reduced pathogenicity on the two rice cultivars used, 3 strains were nonpathogenic, whereas 25 were unaffected in their ability to cause disease. Strains defective in the appressorium assay were also tested in a penetration assay using onion epidermis ***(12)***. The appressorium assay using glass mirror has significantly increased the number of pathogenicity mutants that were identified following generation of the insertion strain library in *M. grisea* strain 70-15 and will be described in detail in the following subheadings.

2. Materials

2.1. A. tumefaciens*-Mediated Transformation Using* Agrobacterium *Strain AD973*

1. Complete media (CM ***[13]***) (per 1 L): 10 g of glucose, 2.0 g of peptone, 1.0 g of yeast extract, 1.0 g of casamino acids, 1 mL of trace elements (*see* **Subheading 2.1.**, **item 10**), 1 mL of vitamin supplement (*see* **Subheading 2.1.**, **item 11**), 50 mL of nitrate salts (*see* **Subheading 2.1.**, **step 12**), pH 6.5 with NaOH, and 15 g of agar.
2. 25X *Agrobacterium* broth (AB) buffer (per 500 mL): 48 g of $K_2HPO_4 \cdot 3H_2O$, and 14.4 g of $NaH_2PO_4 \cdot H_2O$. Store sterile at room temperature.
3. 50X AB salts (per 100 mL): 5.0 g of NH_4Cl, 1.5 g of $MgSO_4$, 10 mL of 1 *M* KCl, 0.7 mL of 0.5 *M* $CaCl_2$, and 5.0 mL of 10 m*M* $FeSO_4$ (*see* **Note 1**). Store sterile at room temperature.
4. AB liquid medium (per 1 L): 3.85 g of $K_2HPO_4 \cdot 3H_2O$, 1.15 g of $NaH_2PO_4 \cdot H_2O$, 1.0 g of NH_4Cl, 2.0 mL of 1 *M* KCl, 1.0 mL of 10 m*M* $FeSO_4$. Sterilize by autoclaving in 100-mL aliquots, then add 7 µL of 1 *M* $CaCl_2$, 125 µL of 1 *M* $MgSO_4$ and 1.0 mL of 20% (v/v) glucose (*see* **Note 2**).
5. AB solid medium (per 500 mL). To 465 mL of double-distilled sterile water (ddH_2O), add 1.0 g of glucose and 7.5 g of agar. After sterilization by autoclaving, allow the solution to cool to 55–60°C and then add: 10 mL of 50X AB Salts, 25 mL of 25X AB buffer, tetracycline to 10 µg/mL, and carbenicillin to 60 µg/mL.
6. 25X AB MES pH 5.8 buffer (per 100 mL): 13.3 g of MES, 9.6 g of $K_2HPO_4 \cdot 3H_2O$, and 2.9 g of $NaH_2PO_4 \cdot H_2O$. Adjust pH to 5.8 with H_3PO_4.
7. AB MES pH 5.8, also known as induction medium (for 150 mL). Combine the following sterile solutions: 139.5 mL of ddH_2O, 6.0 mL of 25X AB MES buffer, pH 5.8, 3.0 mL of 50X AB salts, 1.5 mL of 20% (v/v) glucose (*see* **Note 3**).
8. AB MES pH 5.8 solid induction medium (for 500 mL): 465 mL ddH_2O, 9.0 g of agar. After sterilization by autoclaving, allow the solution to cool to 55 to 60°C and then add 20 mL of 25X AB MES buffer pH 5.8, 10 mL of 50X AB salts, and 5.0 mL of 20% (v/v) glucose.

9. Sucrose selection media (per 1 L): 100 g of sucrose, 3.0 g of yeast extract, 3.0 g of casein hydrolysate enzymatic (N-Z-amine), and 15 g of agar. After sterilization by autoclaving, allow the solution to cool to 55 to 60°C and then add hygromycin B to 350 μg/mL and kanamycin to 100 μg/mL (*see* **Note 4**).
10. Trace elements (for 100 mL): 80 mL of ddH_2O, 2.2 g of $ZnSO_4·7H_2O$, 1.1 g of H_3BO_3, 0.5 g of $MnCl_2·4H_2O$, 0.5 g of $FeSO_4·7H_2O$, 0.17 g of $CoCl_2·6H_2O$, 0.16 g of $CuSO_4·5H_2O$, 0.15 g of $Na_2MoO_4·2H_2O$, and 5.0 g of Na_2-ethylene diamine tetraacetic acid. To make the trace elements stock solution add compounds in order, bring to boil, cool to 60°C, and pH to 6.5 with KOH. Adjust volume to 100 mL. Store at 4°C.
11. Vitamins supplement (for 1 L): 0.001 g of biotin, 0.001 g of pyridoxin, 0.001 g of thiamine, 0.001 g of riboflavin, 0.001 g of *p*-aminobenzoic acid, and 0.001 g of nicotinic acid. Sterilize by filtration and store in a dark glass bottle at 4°C.
12. Nitrate salts (for 1 L): 120 g of $NaNO_3$, 10.4 g of KCl, 10.4 g of $MgSO_4·7H_2O$, and 30.4 g of KH_2PO_4. After sterilization by autoclaving, store at 4ºC.
13. ddH_2O.
14. Carbenicillin: 100 mg/mL stock solution.
15. Hygromycin B: 100 mg/mL stock solution.
16. Kanamycin: 100 mg/mL stock solution.
17. Miracloth™ (Calbiochem, cat. no. 475855).
18. Thomas Scientific black filter papers (cat. no. 4740C10).
19. Glass beads (3 mm, soda lime glass).

2.2. A. tumefaciens*-Mediated Transformation Using* Agrobacterium *Strain AGL1/pBHt-2*

1. 25X AB buffer: *see* **Subheading 2.1.**, **item 2**.
2. 50X AB salts: *see* **Subheading 2.1.**, **item 3**.
3. AB solid medium: *see* **Subheading 2.1.**, **item 5**. Use 50 μg/mL kanamycin for selection instead of carbenicillin and tetracycline.
4. 25X AB MES pH 5.8 buffer: *see* **Subheading 2.1.**, **item 6**.
5. AB MES pH 5.8 solid induction medium: *see* **Subheading 2.1.**, **item 8**. After sterilization by autoclaving, allow the solution to cool to 55 to 60°C and then add the components described in *see* **Subheading 2.1.**, **item 8**. Also, add acetosyringone (filter sterilized) to 200 μg/mL (*see* **Notes 5–7**).
6. Sucrose selection media: *see* **Subheading 2.1.**, **item 9**. Substitute 200 μ*M* of cefotaxime in place of kanamycin, and add hygromycin B to 350 μg/mL**.**
7. Minimal medium (per 1 L). Combine the following sterile solutions: 941.5 mL of ddH_2O, 10 mL of K-buffer pH 7.0 (200 g/L K_2HPO_4, 145 g/L KH_2PO_4), 20 mL of M-N (30 g/L $MgSO_4·7H_2O$, 15 g/L NaCl), 1.0 mL of 1% (w/v) $CaCl_2·2H_2O$, 10 mL of 20% (w/v) glucose, 10 mL of 0.01% (w/v) $FeSO_4$ (filter sterilize), 5.0 mL of spore trace elements (100 mg/L $ZnSO_4·7H_2O$, 100 mg/L $CuSO_4·5H_2O$, 100 mg/L H_3BO_3, 100 mg/L $MnSO_4·H_2O$, 100 mg/L $Na_2MoO_4·2H_2O$), and 2.5 mL of 20% (w/v) NH_4NO_3.

8. AGL1 induction medium (per 1 L). Combine the following sterile solutions: 898 mL of ddH_2O, 0.8 mL of 1.25 *M* K-buffer pH 4.9 (184 g/L K_2HPO_4, adjust pH to 4.9 with phosphoric acid), 20 mL of M-N solution (*see* **Subheading 2.2., step 7**), 1.0 mL of 1% (w/v) $CaCl_2$-2H_2O, 10 mL of 0.01% (w/v) $FeSO_4$, 5.0 mL of spore trace elements (*see* **Subheading 2.2., step 7**), 2.5 mL of 20 % (w/v) NH_4NO_3, 10 mL of 50 % (w/v) glycerol, 40 mL of 1 *M* MES pH adjusted to 5.5 with NaOH (filter sterilize and store at –20°C; *see* **Note 8**), 10 mL of 20% (w/v) glucose. After sterilization by autoclaving, allow the solution to cool to 55–60°C and then add 2 mL of 100 m*M* acetosyringone (3',5'-dimethoxy-3'-hydroxy-acetophenone) in ethanol.
9. Miracloth™ (Calbiochem, cat. no. 475855).
10. ddH_2O.
11. Kanamycin: 100 mg/mL stock solution.
12. Thomas Scientific black filter papers (cat. no. 4740C10).
13. Acetosyringone: 100 m*M* stock solution, dissolved in ethanol.
14. Glass beads (3-mm soda lime glass).
15. Hygromycin B: 100 mg/mL stock solution.
16. Cefotaxime: 200 m*M* stock solution.

2.3. Processing and Storage of M. grisea Insertion Strain Lines

1. Oatmeal agar medium (OA) (per 1 L): 500 mL of ddH_2O, 50 g of old-fashioned oats (e.g., Quaker®). Combine in a 1-L Erlenmeyer flask and place in a 70°C water bath for 1 h mixing occasionally. Strain the solution through a double layer of cheesecloth (*see* **Note 9**). Adjust the volume to 1 L with ddH2O and add 13.5 g of agar. Sterilize by autoclaving for 30 min. After sterilization by autoclaving, allow the solution to cool to 55 to 60°C and then add hygromycin B to 300 μg/mL and bacterial counter selection (*see* **Note 10**).
2. CM: *see* **Subheading 2.1., step 1**. When antibiotic selection is required sterilize by autoclaving, allow the solution to cool to 55 to 60°C and then add hygromycin B to 200 μg/mL.
3. Pasteur pipets, flint glass, 14.6 cm (Fisher brand cat. no. 13-678-6A) were heated to round up the tip before streaking conidia.
4. Very-fine-point forceps (VWR cat. no. 25607-856).
5. Single-use syringes, 3-mL vol, 25-gage, 1.5-in. (VWR cat. no. BD309582). The syringes were sterilized by autoclaving and reused.
6. Desiccant activated silica gel (6-12 mesh; Eagle Chemical Company, cat. no. MIL-D-3716A).
7. Filter paper no. 597 (S & S Biopath Inc., cat. no. 10318893). Pieces of filter paper were generated using a hole-punch and sterilized by autoclaving before use.

2.4. In Vitro Appressorium Formation Assay Using Glass Mirror

1. OA: *see* **Subheading 2.3., step 1**, except no antibiotic selection is required so do not add hygromycin B or bacterial counter selection.

2. 0.25% Gelatin (for 100 mL): Add 0.25 g of gelatin directly to a bottle, add 100 mL of ddH_2O, and sterilize by autoclaving.
3. Pilkington eclipse bluegreen reflective glass (Pilkington PLC, UK) purchased from Tucson Glass and Mirror, Tucson, AZ as plates 15.5 × 11.4 × 0.5 cm, which are large enough to screen 24 fungal strains.
4. Large Tupperware™ containers.
5. Cover slips.

3. Methods

3.1. Transformation of M. grisea *Strain 70-15*

3.1.1. A. tumefaciens*-Mediated Transformation Using* Agrobacterium *Strain AD973*

3.1.1.1. Preparation of *Agrobacterium* Cells

1. Streak *Agrobacterium* strains EHA105 and AD973 (EHA105/pAD1624) from a glycerol stock onto AB solid medium to obtain single colonies (**Fig. 1**). EHA105 is a Km^S derivative of EHA101, where the kanamycin marker was deleted by recombination ***(8)***. EHA101 is a C58-derivative strain where the resident Ti plasmid was replaced with a modified pTiBo542 supervirulent plasmid, pEHA101 ***(14)***. pEHA101 contains a Km^R marker in place of the T-DNA region and is, thus, T-DNA minus. For AD973 the AB solid medium should be supplemented with carbenicillin (60 μg/mL) to select for maintenance of the T-DNA-containing plasmid. Incubate at 28°C for 48 h (*see* **Note 11**).
2. Pick a single colony and streak it onto AB solid medium to obtain confluent growth. For AD973, the media should be supplemented as above. Incubate at 28°C for 48 h.
3. Transfer one-third of the cells from the AB solid medium plate to 2.0 mL of AB liquid medium (*see* **Note 12**). For AD973 the media should be supplemented with carbenicillin (60 μg/mL). The starting culture should be slightly turbid. Incubate at 28°C with 200-rpm aeration for 18 to 24 h (cultures should reach an $OD_{600} > 1$).
4. Transfer 300 μL of the overnight culture to 5.0 mL of AB MES pH 5.8 induction medium. For AD973 the media should be supplemented with carbenicillin (60 μg/mL). Incubate at 28°C with 200-rpm aeration for 18 to 24 h.
5. Measure the OD_{600} in a spectrometer (it should be between 0.5 and 1.0). 1.0 OD_{600} equals 1×10^9 cells/mL.
6. Pellet the remaining *Agrobacterium* cells by centrifugation at 3400*g* for 10 min at room temperature.
7. Pour off the supernatant and resuspend the bacterial cells in AB MES pH 5.8 medium to obtain a final concentration of 1×10^9 cells/mL. Further dilute to 1×10^8 cells/mL by adding 100 μL of the bacteria to 900 μL of AB MES, pH 5.8, buffer in a microcentrifuge tube.

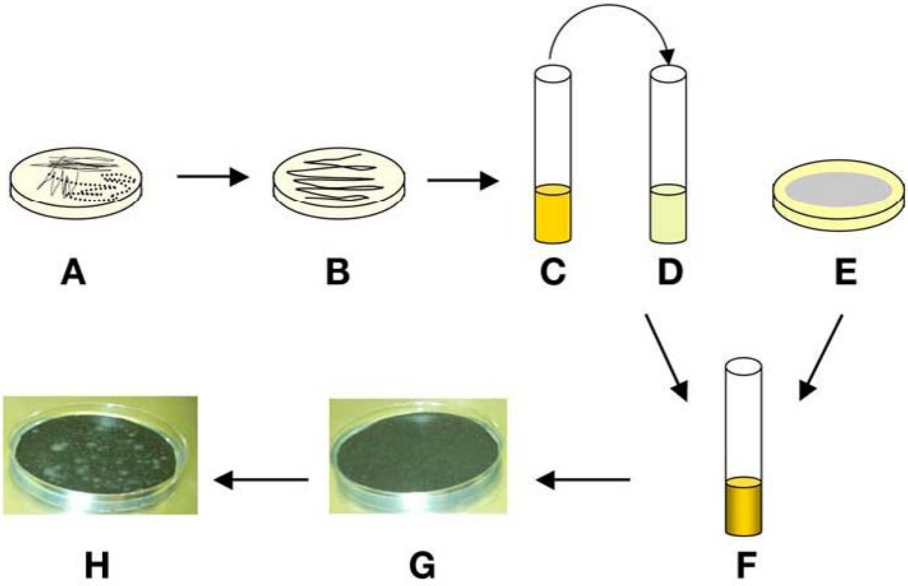

Fig. 1. **(A)** Streak *Agrobacterium tumefaciens* strains from glycerol stocks onto *Agrobacterium* broth (AB) medium with selection. **(B)** Restreak from a single colony onto AB medium with selection to prepare cells for liquid inoculum. **(C)** Transfer a heavy inoculum of cells to AB liquid medium with selection and grow to OD_{600} greater than 1.0. **(D)** Dilute the culture and grow in AB MES pH 5.8 liquid medium for *vir* gene expression. Harvest and suspend at 1×10^8 cells/mL. **(E)** Harvest *Magnaporthe conidia*, count and dilute to 1×10^6 conidia/mL. **(F)** Mix conidia and bacterial cells for co-cultivation. **(G)** Spread co-cultivation mixture on black filter papers, premoistened on AB MES pH 5.8 agar plates with no selection. Incubate for 48 h at 28°C. **(H)** Transfer filters to plates containing hygromycin B for selection of transformants and antibiotic (kanamycin or cefotaxime) for selection against *Agrobacterium*.

3.1.1.2. Preparation of Fungal Conidia

1. *M. grisea* strain 70-15 was inoculated onto CM agar plates and grown for 8 d at 25°C, under constant fluorescent light. Alternatively, conidia can be generated by inoculation onto oatmeal agar plates and grown in the same manner.
2. Harvest the conidia from the fungal plate culture by adding 3.0 mL of ddH_2O and gently rubbing the surface of the mycelium with a glass spreader.
3. Filter the spores through Miracloth™ into a 15-mL Falcon tube (model no. 2059) and rinse the cloth with 2.0 mL of ddH_2O. Dilute the conidia to 1×10^6 conidia/mL.

3.1.1.3. Cocultivation of the Bacterial and Conidial Cells

1. Combine *Agrobacterium* and fungal cells with AB MES pH 5.8 buffer at a ratio of 100 *A. tumefaciens* cells to one *M. grisea* conidium to create the mixture of cells for co-cultivation. Therefore add 100 µL of bacteria (1×10^8/mL) to 100 µL of conidia (1×10^6/mL) and 800 µL of AB MES pH 5.8 buffer (*see* **Note 13**).
2. Place the black filter papers onto the AB MES pH 5.8 solid induction agar plates (with no antibiotic selection) along with 30 to 35 glass beads. Add 200 µL of the co-cultivation mixture and spread immediately (*see* **Notes 14** and **15)**. Incubate for 48 h at 28°C.
3. Transfer the filter papers containing the cocultivation mixture to Sucrose Selection plates supplemented with hygromycin B (350 µg/mL) and with kanamycin (100 µg/mL) to select against *A. tumefaciens* growth. Incubate at 28°C for 5 to 7 d until colonies appear (*see* **Note 16**).

3.1.2. A. tumefaciens*-Mediated Transformation Using* Agrobacterium *Strain AGL1/pBHt-2*

3.1.2.1. Preparation of *Agrobacterium* Cells

1. Streak *A. tumefaciens* strain AGL1/pBHt-2 from the glycerol stock onto AB solid medium supplemented with kanamycin (50 µg/mL) to obtain a single colony (*see* **Note 11**; **Fig. 1**). Incubate at 28°C for 48 h.
2. Pick a colony and streak it onto AB solid medium supplemented with kanamycin (50 µµg/mL). Incubate at 28°C for 48 h.
3. Transfer a third of the cells from the AB solid medium plate into 5.0 mL of minimal liquid medium containing kanamycin (50 µg/mL). The starting culture should be slightly turbid (*see* **Note 12**). Incubate at 28°C with 200-rpm aeration for 48 h.
4. Measure OD_{600} in spectrometer. Transfer the *Agrobacterium* cells to induction liquid media supplemented with kanamycin (50 µg/mL) to a final concentration equivalent to an OD_{600} of 0.15. Incubate this culture at 28°C with 200-rpm aeration for 6 h (note final OD_{600} will be approx 0.25, equal to 2.5×10^8 cells/mL).

3.1.2.2. Preparation of Fungal Conidia

See **Subheading 3.1.1.2.** for details.

3.1.2.3. Cocultivation of the Bacterial and Conidial Cells

1. Combine the *Agrobacterium* and fungal cells with induction media at a ratio of 250 *A. tumefaciens* cells to one *M. grisea* spore. Therefore add 100 µL of bacteria to 100 µL of conidia and 800 µL of induction media (*see* **Note 13**).
2. Place the black filter papers onto AB MES pH 5.8 solid medium supplemented with acetosyringone (200 µg/mL) along with 30 to 35 glass beads. Add 200 µL of the co-cultivation mixture and spread on the filter paper (*see* **Notes 14** and **15)**. Incubate for 48 h at 28°C.
3. Transfer the filter papers containing the co-cultivation mixture to Sucrose Selection plates containing hygromycin B (350 µg/mL) and cefotaxime (200 µ*M*) to

select against *Agrobacterium*. Incubate at 28°C for 5 to 7 d until colonies appear (*see* **Note 16**).

3.2. Processing and Storage of M. grisea *Insertion Strain Lines*

1. After 5 to 7 d, pick hygromycin B-resistant *M. grisea* colonies from the black filter paper using sterile forceps and transfer them to 24-well plates containing OA supplemented with hygromycin B to 300 μg/mL and kanamycin to 100 μg/mL (*see* **Note 17**). Incubate at 25°C, under constant fluorescent light for 5 to 7 d to allow the fungus to sporulate.
2. Streak conidia using modified Pasteur pipets onto CM supplemented with hygromycin B to 300 μg/mL and bacterial counter selection (*see* **Notes 10** and **18**). Incubate at 28°C overnight (15–18 h).
3. Use a dissecting microscope to view the conidia from each insertion strain and pick an individual germinated conidium using a 1-mL syringe (*see* **Notes 19** and **20**). Transfer the conidium to one well of a 24-well plate containing CM supplemented with hygromycin B to 200 μg/mL. Each well of the plate should contain three pieces of filter paper arranged so that the conidium can be placed in the center of the well. Incubate at 25°C, under constant fluorescent light for 7 d.
4. Peel the filter papers using forceps and transfer to 96-well plates so that there are three replica 96-well plates each containing one of the filter papers covered with mycelium, which regenerated from an individual conidium (*see* **Note 21**).
5. Place the 96-well plated in an airtight unit containing silica gel for 7 d at room temperature to allow the filters to desiccate. Transfer the plates to airtight containers with silica gel, place the container in a plastic bag and store at –20°C.

3.3. In Vitro Appressorium-Formation Assay Using Glass Mirror

1. Harvest conidia from a colony growing on an OA plate by adding 35 μL of 0.25% gelatin to the surface of the culture (*see* **Note 22**). Draw the gelatin up and then pipet up and down once more to ensure enough conidia for the assay (*see* **Note 23**). Place spore suspension onto the glass mirror (*see* **Notes 24** and **25**).
2. Incubate the glass mirror in a moist chamber. After 6 h, add cover slips to the spore suspension drops and score for appressorium development after 24 h (*see* **Note 26**).
3. Assess a random sample of 20 conidia scoring whether they have germinated, formed appressoria or have any interesting spore morphology phenotypes. For each assay, results are compared to the parental strain 70-15.
4. Insertional mutants that show a difference from wild type are assessed in two further appressorium assays.
5. Lines defined as appressorium variants after three assays are tested in rice seedling pot infection assays on cultivars M-202 and Maratelli to screen for defects in pathogenicity.

4. Notes

1. 50X AB salts should be mixed well before each use.

2. AB liquid medium may precipitate but do not mix before use.
3. AB MES pH 5.8 induction medium should be stored for only 1 mo, after which time the pH of the solution should be determined before further use.
4. Sucrose selection media should not be stored for more than 1 mo.
5. AB MES pH 5.8 solid induction medium should be made fresh before each transformation.
6. Acetosyringone will precipitate from solution during storage, resuspend by placing in a 37°C water bath.
7. It is necessary to induce *Vir* gene expression in the *A. tumefaciens* strain AGL1/pBHt-2, by the exogenous addition of acetosyringone, as the binary vector pBHt-2 does not contain a constitutive *virG* gene.
8. 1 *M* MES pH 5.5 can precipitate, place the bottle in a 65°C water bath to resuspend.
9. Ensure the oats are well suspended before straining them through cheesecloth, which is laid over a large funnel. Carefully gather the edges of the cheesecloth and twist to strain out most of the liquid from the oats.
10. For strains transformed with AD973, include kanamycin (100 µg/mL) as counter selection and for those transformed with AGL1/pBHt-2, use cefotaxime (200 µ*M*).
11. Glycerol stocks are prepared by growing a bacterial culture in AB medium (plus appropriate antibiotic) then adjusting the volume to contain 20% glycerol before storage at –80°C.
12. It is not necessary to count the number of *Agrobacterium* cells, which are transferred to the AB liquid media. There should be a sufficient number to render the liquid media turbid but small variations will not affect the overall yield of bacterial cells.
13. *A. tumefaciens*-mediated transformation of *M. grisea* was optimized by testing various cell ratios to find the one that produced the maximum number of transformed lines ***(1)***. This optimization should be undertaken before transformation with an alternative *Agrobacterium* strain and/or a different fungus.
14. Ensure the black filter papers are placed on the co-cultivation plates and allowed to hydrate before adding the co-cultivation mixture to them. It is important to dispense the co-cultivation mixture promptly on the filter papers. Gently mix the cells before dispensing each aliquot.
15. As the black filter papers will readily absorb the co-cultivation mixture it is essential to add the bacterial and fungal cells and then spread them immediately over the plate. Concentrated areas of cells make selection of transformants difficult.
16. After *M. grisea* transformation by *A. tumefaciens*, it is possible to pick hygromycin B resistant fungal lines after 5 d. If the maximum number of transformants is required selection plates can be incubated at 28°C for another 2 d when further transformed strains may have developed. However, after this amount of time there may be considerable background growth on the selection plates, which will interfere with the selection of individual colonies.
17. Poke the forceps through the filter paper and transfer some of the paper with the fungal colony. It will not interfere with growth of the fungus. Only a small sample

of the colony should be removed to reduce the possibility of transferring more than one fungal isolate.

18. Divide the Petri plate into 12 sections and streak each one with a different fungal insertion line.
19. Use the needle point of the syringe to carefully cut a small square of agar surrounding the conidium and then insert the needle under the conidium at an angle to lift out the agar plug.
20. *M. grisea* insertion lines that failed to produce a hygromycin B-resistant monoconidial were stored in the form of the original mycelium from the 24-well OA plate from which conidia had been taken. These represented potentially interesting insertional mutant strains; the mycelium was able to grow in the presence of hygromycin B, suggesting successful *Agrobacterium* transformation, but individual conidia could not grow under selection suggesting that a gene essential for growth or differentiation of the fungus may have been disrupted.
21. Filter papers of the *M. grisea* insertion strain lines were generated and stored in triplicate. One 96-well plate remained at the laboratory where the insertion lines were generated, a second plate was sent to North Carolina State University, where pathogenicity assays were undertaken in the laboratory of Dr. Ralph Dean and the final plate was sent to the Fungal Genetics Stock Center for storage and distribution to the fungal community.
22. If the appressorium assay is to be conducted in a high-throughput manner, 24-well OA plates can be used for growth of the fungal isolates that are going to be tested. Obtain glass mirrors that are larger than the 24-well plate so that there is sufficient room to add 24 cover slips to the conidial droplets.
23. It is important not to touch the tip of the pipet to the surface of the OA plate to avoid disturbing the mycelium as mycelial fragments interfere with the assay.
24. If the fungal strain you are testing in the appressorium assay conidiates poorly it may be necessary to pipet the 0.25% gelatin solution up and down several more times to obtain sufficient conidia for the assay.
25. Test both sides of the glass mirror as one side has an enhanced mirror-effect and use of this side results in more consistent germination of the fungus.
26. Tupperware containers are effective for incubation of glass mirrors. Add several layers of paper towel, which are then saturated in water. The container needs to be airtight to prevent the drops of spore suspension from evaporating.

Acknowledgments

This project was funded by the National Science Foundation Plant Genome Program, award DBI no. 0115642. We thank Dr. Jin-Rong Xu from Purdue University for the AGL1 strain and Dr. Yong-Hwan Lee from Seoul National University for discussions about the in vitro appressorium assay. This project was conducted in collaboration with Dr. Mark Farman of the University of Kentucky, whose laboratory produced half of the insertion mutant library and Dr. Ralph Dean, of North Carolina State University.

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7

Identification of Components in Disease-Resistance Signaling in *Arabidopsis* by Map-Based Cloning

Yuelin Zhang, Jane Glazebrook, and Xin Li

Summary

With the whole genome sequence and thousands of defined polymorphisms between ecotypes available, it has become much easier to clone a gene by position (map-based cloning) in Arabidopsis. Recent development of DNA-isolation methods in plants also dramatically facilitated large-scale processing of DNA samples. Here, we describe detailed protocols for each step on general scheme of map-based cloning, from mutagenesis to genetic analysis, from rough mapping to fine mapping, and at the end to cloning the gene. Not only can these methods be used to isolate genes that are involved in plant innate immunity, they can also be adapted for any forward genetics projects in Arabidopsis.

Key Words: Forward genetics; positional cloning; map-based cloning; mapping; plant disease resistance; plant innate immunity; mutagenesis.

1. Introduction

Ideally, if a collection of sequence-indexed homozygous knockout mutants for all *Arabidopsis* genes were available, similar to what has been achieved in yeast, we could simply use the collection for mutant screens to quickly associate genes with mutant phenotypes of interest. Unfortunately, such a mutant collection for *Arabidopsis* remains years away. Furthermore, certain mutant phenotypes can only be observed in specific genetic backgrounds, such as those identified in suppressor or enhancer screens. Thus, forward genetics will still play a major role in *Arabidopsis* research even if knockout mutants for most genes become available. With the complete *Arabidopsis* genome sequence known ***(1)*** and a large collection of sequence polymorphisms between the Columbia and Landsberg accessions available ***(2)***, it is now taking much less time and effort to clone a gene based on its position. This chapter shares the

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

protocols for mutant screening and map-based cloning that have been used in our labs to identify genes involved in disease resistance signaling.

2. Materials

1. Ethane methyl sulfonate (EMS)
2. Fast neutron source
3. Polymorphism markers for mapping. Examples include simple-sequence length polymorphism, insertion/deletion polymorphisms (InDel), codominant cleaved amplified polymorphic sequences, and single nucleotide polymorphism.
4. Oligonucleotide primers for sequencing, polymerase chain reaction (PCR) cloning, etc.
5. Taq DNA polymerases.
6. Restriction enzymes.
7. Agarose gel electrophoresis apparatus.
8. FTA® classic cards (Whatman).
9. Micro-punch kit and replacement parts for FTA cards (Whatman).
10. FTA wash solution: 10 m*M* Tris-HCl, 2 m*M* ethylene diamine tetraacetic acid, 0.1% Tween-20, pH 8.0.
11. TE-1 wash buffer: 10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA.
12. Binary BAC or TAC clones.

3. Methods

The methods outlined in this section describe the general procedures in forward genetics including mutagenesis, mutant screening, crude mapping, fine mapping, mutation identification by sequencing, and complementation.

3.1. Mutagenesis and Building a Mutagenized Population

Before beginning a forward genetics project, it is important to choose an appropriate genetic background for the mutant screen. In Arabidopsis, the Columbia (Col) ecotype is fully sequenced and has been used most frequently for mutant screening. Landsberg erecta (Ler) is another widely used ecotype. Mutations that can be mapped on a Col × Ler cross are the easiest to identify by positional cloning because of the wealth of polymorphism information for these two accessions. If a suppressor or an enhancer screen is planned, the genetic background is necessarily that of the original mutant.

3.1.1. Choice of Mutagens (see ***Note 1***)

The most commonly used mutagen in Arabidopsis is EMS. Almost all EMS-induced mutations are G/C to A/T transitions. Because EMS is a highly effective mutagen that induces mutations randomly in the genome ***(3)***, a relatively small population of a few thousand lines is enough to recover loss-of-function mutations in most genes. Gain-of-function mutations can also be obtained by EMS mutagenesis, although at a lower frequency. For EMS mutagenesis, we

normally treat approx 5000 *Arabidopsis* seeds with 20 m*M* EMS for 18 h. A detailed EMS mutagenesis protocol can be found at ftp://ftp.arabidopsis.org/home/tair/Protocols/compleat_guide/6_EMS_mutagenesis.pdf. Great care must be taken when working with EMS to avoid exposure of laboratory personnel to the mutagen.

Fast neutrons (FNs) are another frequently used mutagen. Most mutations induced by fast neutrons are small deletions, although rearrangements also are observed. These are almost exclusively loss-of-function mutations. Although the efficiency of FN mutagenesis is approx two- to threefold lower than EMS ***(3)***, one potential advantage is that deletions can be easier to identify once a mutation is mapped to a small region. For mutant screens that are easy to carry out, FN mutagenesis can be a good choice. For *Arabidopsis*, the dose of FN used by most researchers is 60 Gy. FN mutagenesis does require accessibility to a cyclotron that generates reliable FN. We have successfully used a facility through Joe Palfalvi (palfalvi@sunserv.kfki.hu; Atomic Energy Research Institute, Budapest, Hungary).

3.1.2. Pool Size of Mutagenized Populations

Large pools (usually approx 100 M1 lines per pool) usually are used for mutant screening to ease collection and handling of the mutant population, but small pools work best for screens that are not very laborious. Although it takes a little more effort to build the mutagenized population, it often is easier to determine whether the mutations are dominant or recessive, and the mutants found in each pool almost always come from a single original mutation. For example, in the primary screen for suppressors of *snc1*, where we were searching for big mutants among crowds of small sneaky *snc1* plants, smaller pools helped us to rapidly identify the dominant mutants in the M2. These mutations were not interesting to us, as they are almost exclusively *SNC1* revertants ***(4)***. To screen for suppressors of *snc1*, we pooled seeds from 10 fast neutron-treated M1 lines into one pool and planted approx 200 M2 seeds from each pool for screening. On average, each M1 line is represented by about 20 M2 seeds. If a dominant mutation occurred in an M1 plant, usually more than 10 mutant plants are found in the M2s. For recessive mutants, the number of plants in each pool that display the mutant phenotype is significantly smaller. As a result, we were able to weed out a large number of revertants in the primary screen.

3.2. Mutant Screening and Genetic Analysis

The screening process is dependent on the phenotypes being assayed. Ideally, the primary screen is performed in a high-throughput fashion. Sometimes the primary screen can be designed based on a secondary phenotype that can be easily detected, whereas the target phenotype can be analyzed in the secondary screen. For example, to screen for suppressors of *snc1*, we took

advantage of the secondary developmental phenotypes caused by constitutive activation of defense responses, namely small stature and curly dark green leaves. In the primary screen, we simply looked for mutants that were bigger than *snc1*, so we were able to go through about 200,000 M2 plants quickly ***(5)***. In the secondary screen, the selected mutants were assayed for loss of *PR-2* gene expression and resistance to pathogens.

Once a mutant is obtained, a few standard Mendelian genetic tests usually are conducted. Two crosses almost always have to be performed: a backcross with the starting line that the mutant was derived from, and a mapping cross with another accession. The backcross is used to determine the number of mutations that are responsible for the mutant phenotype and whether the mutation is dominant or recessive. The mapping cross is used to establish an F2 population for mapping. Frequently a single mutation is responsible for the mutant phenotype observed, and most mutations caused by EMS or FN are recessive.

In suppressor and enhancer screens, the mutants recovered may not have detectable phenotypes when they are in the wild-type background. In this case, it may be necessary to introgress the original mutation into another ecotype and use the introgressed line for the mapping cross. For example, to map the *mos* (*modifiers of snc1*) mutants, we introgressed the original *snc1* mutation (from Col) into the Ler ecotype (named Ler-*snc1*) through repetitive backcrossing with Ler, and selection of *snc1* homozygous plants in the F2 ***(5)***. Theoretically, after six backcrosses, approx 98.4% of the genome is Ler, with the exception of regions around the original mutation that remain Col. Mapping was conducted on the F2 progeny of the mapping cross between the *mos snc1* suppressor mutants and Ler-*snc1*. This kind of introgression can also be assisted using genome-wide markers to reduce the number of generations required to accomplish the introgression.

3.3. Rough Mapping

Rough mapping is normally conducted through linkage analysis using homozygous mutant plants selected from the F2 mapping population. In principle, any F2 plants of known genotype at the gene of interest can be used. Because most mutations are recessive, usually only the phenotypes of the homozygous mutant plants are known. The genotypes of homozygous mutant plants from the F2 mapping population are then determined at many loci throughout the genome. Linkage between the mutation of interest and a nearby molecular marker is evident from segregation ratios that differ from the expected 1:2:1. If the mutation was isolated in Col background, and the mapping cross was with Ler, then the segregation of linked markers will be skewed in favor of Col alleles with a reduction in the number of Ler alleles. Statistical

Table 1
A Set of InDel Markers for Rough Mapping Using Col and Ler

Chromosome	Position (MB)	BAC location	Size of Col band (bp)	Primer sequences (5'-3')
1	8	F19G10	467	F: atgtcaccgtgaacgacatc R: tgcgagttaagacctaggag
1	25.3	T2E12	531	F: cgactagccagtccgataca R: cgttttgggagccacgtttc
2	9.2	F2G1	411	F: cgtcgtcggaagtttcagag R: gaataagaagaacacatgcgtc
2	12.3	T8O18	702	F: gatatggatgtaacgacccaa R: cagcttcgagtggattctac
3	5	MIE1	449	F: ctaagttcttccaccatctg R: caaggagcatctagccagag
3	20	F24B22	433	F: ctgggaacaaaggtgtcatc R: caaggtctccagaacacaaac
4	6.5	T4C9	648	F: caaaggtttcgtgtcggagc R: cgttgacgggatactcggtg
4	12.9	T13J8	333	F: atgttcccaggctccttcca R: gagatgtgggacaagtgacc
5	7.8	MYJ24	569	F: ctaatcccaagctgaatcac R: tgacagagaatccgactgtg
5	19.4	K19E20	620	F: gacaagaaccacatgagagc R: gttatgtgtacacttcaggtc

The sizes of the fragments are between 200 and 700 bp, and the PCR conditions using regular Taq polymerase for all of them are 94°C for 2 min followed by 40 cycles of 94°C at 15 s, 55°C at 30 s, and 68°C at 1 min. All polymorphisms can be resolved using 1% agarose gels. For all the markers, the Col fragments are larger than the Ler ones.

significance of apparent linkage should be tested using chi-squared analysis. **Table 1** lists a set of good InDel markers designed based on the list of Col/Ler polymorphisms provided by Monsanto ***(2)***. These markers were used routinely for our rough mapping experiments. We found that 24 plants homozygous for a recessive mutation are often enough to define the chromosome arm where the mutation resides, whereas bigger populations help narrow the region down.

Once a mutation is found to be linked to a marker, the next step is to identify two flanking markers, one on each side of the mutation. This is one of the most important steps in cloning a gene based on its map position. In cases in which rough mapping is carried out on plants homozygous for the mutation, an indication that flanking markers have been identified is that the recombinants between marker one and the gene of interest and marker two and the gene of interest are

mutually exclusive. This is because two recombination events in a small interval are required to generate a plant that is homozygous Col at the mutation of interest and heterozygous at each of two flanking markers, and such double recombinants are very rare. The flanking markers can be subsequently used for fine mapping in order to collect additional recombinants for chromosome walking.

For crude mapping to be successful, it is crucial that the homozygous mutant plants are identified correctly. If there is any uncertainty in identification of homozygous mutant plants in the F2, it is advisable to verify phenotypes in the F3. If any plants judged to be homozygous mutant are actually heterozygous or wild-type, then they will appear to be recombinants between close makers and the gene of interest. This error introduces a great deal of noise into the mapping data, and can make it impossible to find the map position.

3.4. Fine Mapping

The purpose of fine mapping is to gather additional recombinants so that the mutation can be narrowed down to a region of approx 100 kb or less. Several techniques such as complementation with tansgenes, DNA sequencing, and examination of sequence-indexed transfer DNA (T-DNA) lines can then be used to identify the gene of interest within this interval. In our experience, a mutation can normally be mapped to a region smaller than 100 kb using a fine mapping population of approx 800 plants.

3.4.1. High-Throughput Genotyping Using FTA Technology and PCR

Fine mapping requires PCR-genotyping of a large number of plants, so DNA extraction from individual F2 plants can become very time-consuming. Recently, an FTA technology has been adapted for plant material that dramatically speeds up the fine mapping process ***(6)***. The FTA technology skips the whole DNA extraction step, and instead the leaf tissue is printed onto FTA paper for direct washing and PCR. Here is the FTA protocol we routinely use for fine mapping:

1. Grow up the fine mapping population (F2 individuals from the mapping cross) until the homozygous mutant plants can be identified based on their phenotypes.
2. Pick the individuals with the mutant phenotype and transplant them into inserts that can be easily numbered.
3. Number the plants, cut one small leaf from each plant, and print it onto FTA paper. To print the leaf, place it on the FTA paper, cover it with a piece of Parafilm and press firmly with the end of a centrifuge tube or similar object. You should see a green smudge on the paper, but the paper itself should not be damaged. FTA paper is available in a number of sizes. A piece the size of a 96-well plate can be used to print 96 samples arranged in the same way as a 96-well PCR plate. The prints need to be air-dried for at least 30 min before proceeding.

4. In each well of a 96-well plate, add 50 μL of FTA wash solution.
5. From each leaf print, a small piece of print is punched out using a 1.2 mm Harris Micro-punch (Fisher 09 923 352 is supplied with a convenient cutting mat) and directly ejected into the FTA wash solution in the PCR well. We do not usually experience noticeable cross-contamination between samples using properly dried paper. However, if this becomes a problem, it can be remedied by wiping the punch with 70% ethanol.
6. Let the plate sit at room temperature for 5 min.
7. Remove the FTA wash solution thoroughly with a multichannel pipetman. Make sure all the paper discs stay inside the wells and are not stuck to the pipet tips. To conserve tips, we use the same set of tips to remove the solution from all the wells. We do not usually experience cross-contamination as a consequence.
8. Add 200 μL of TE-1 solution to each well to wash paper, again using a multichannel pipetman. Let the plate sit at room temperature for 5 min.
9. Remove the TE-1 completely.
10. Repeat **steps 8** and **9**. After the last wash, it is important to remove all of the TE-1. It can be helpful to spin the plate briefly in a centrifuge to collect any remaining TE-1 at the bottom of the wells, and then remove it with a multichannel pipet. Now the paper is ready to be used as a DNA template for PCR.
11. The PCR protocol we use employs standard PCR conditions for regular Taq polymerase.
12. The PCR products are loaded on an agarose gel for electrophoresis. It is easier to use combs with well spacing that matches the spacing on multichannel pipets, so that the gels can be loaded with a multichannel pipet.

*3.4.2. Creating Markers for Chromosome Walking (see **Note 2**)*

Once the fine mapping population has been genotyped with the two flanking markers, progressively closer markers are used on the recombinants to narrow the interval containing the mutation. There are a large number of markers available from the TAIR website (http://www.arabidopsis.org/servlets/Search?action=new_search&type=marker) that may be useful. With the genome sequence known, it is also relatively easy to design new markers if necessary.

We found that the easiest markers to use are the InDel or simple-sequence length polymorphism. A large collection of sequence polymorphisms between Col and Ler ecotypes and also the Landsberg genome sequences can be downloaded from the TAIR website (http://www.arabidopsis.org/Cereon/index.jsp).

3.5. Identification of the Gene of Interest

Once the position of a mutation has been defined within a 100-kb interval, the gene can be identified by DNA sequencing, complementation using a transgene, or examination of sequence-indexed T-DNA insertion lines. Preferably, once one of these methods has been used to identify the gene of interest, the identity of the gene is confirmed using the other methods.

3.5.1. *Identify Molecular Lesions in Mutants by Sequencing*

With the cost of primers and DNA sequencing decreasing, it is now possible to routinely sequence a 100-kb region to find a mutation. Sequencing is performed using DNA fragments amplified from the genomic DNA of the mutant as templates. Overlapping fragments are sequenced to cover the region between the two final flanking markers. To reduce the cost, one could start sequencing the coding regions first, because most defects are in coding regions. Once the raw sequence data is obtained, BLAST2 can be used to pair the sequences from the mutant to the wild type (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Mismatches are potential mutations in the gene of interest. PCR tends to introduce mutations into the amplified products. This is not a problem in the approach described here because the template for sequencing is the PCR product itself, and not a single molecule from a PCR reaction that has been cloned into a plasmid. The resulting sequence is the average sequence of all the PCR products from the amplification reaction. Because there are multiple products even in the first amplification cycle, mutations in individual molecules are not apparent in the DNA sequencing results.

3.5.2. *Complementation* (see ***Notes 3** and **4***)

If the region containing the gene of interest is rather large, or sequencing is inconvenient, an alternative is to use complementation. One way to do this is to produce cosmid libraries in a binary vector from BAC or other large clones spanning the location of the gene of interest. The resulting cosmids are subsequently transferred into *Agrobacterium tumefaciens* and the bacterial strains are used to transform the mutant plants by floral dipping ***(7)***. Cosmids that restore the phenotype to wild-type very likely contain the gene of interest. If several overlapping cosmids complement the mutant phenotype, the gene of interest can be deduced. This gene should then be sequenced from mutant plants to identify the mutation, as described in the previous section.

When a gene has been identified by DNA sequencing rather than complementation, it is advisable to test the ability of the putative gene of interest to complement the mutant phenotype. The wild-type gene can be amplified from the genomic DNA of the wild-type plants by PCR using proofreading enzymes such as Pfx DNA polymerase (Invitrogen) and Phusion™ High-Fidelity DNA polymerase (New England Biolabs). The PCR fragment is then digested with restriction enzymes and cloned into a binary vector. The resulting clone is introduced into mutant plants by *Agrobacterium*-mediated transformation. Alternatively, the wild-type gene can be subcloned from a BAC clone or TAC clone containing the gene. Transformants are then selected and analyzed for complementation of the mutant phenotypes.

3.5.3. Examination of Sequence-Indexed T-DNA Lines

Sequence-indexed T-DNA lines can also be used to identify genes of interest within a small interval ***(8,9)***. T-DNA lines with mutations in each of the genes in an interval can be tested for the mutant phenotype. T-DNA insertion mutants that show the mutant phenotype likely define the gene of interest. Unlike the other two methods previously described, this approach has a fairly high likelihood of failure. Sequence-indexed T-DNA lines are not available for all genes, and not all T-DNA insertions are null mutations. Also, not all genes are correctly annotated, so if the mutation of interest lies in an unannotated or incorrectly annotated gene, it could be missed. Nevertheless, this approach can be very useful, especially if the investigator has a good idea about what sort of gene is likely to be responsible for the mutant phenotype. This approach is also useful for confirmation of a gene defined by sequencing or transgene complementation. A T-DNA insertion in the gene of interest should fail to complement a recessive mutation in that gene.

4. Notes

1. Because EMS is a highly efficient mutagen, fewer plants are required to hit all the genes in the genome in a mutant screen than if other mutagens are used. However, at the same time, more mutations are present in each line, which can sometimes cause problems in phenotyping. Backcrossing is often required to remove the unwanted mutations.
2. Because the Ler sequence was determined by a shotgun approach and sequence accuracy is moderate, not all the polymorphisms indicated are real. A control experiment using the two parental accessions should be carried out to check markers.
3. Sometimes, for reasons such as being too close to the centromere or hitting a cold spot for recombination, it is impossible to narrow the mutation down to a region that is small enough for sequencing. In this case, one can consider narrowing the region down by complementation using overlapping binary BAC or TAC clones covering the region.
4. Although cDNA clones driven by the cauliflower mosaic virus 35S promoter have been used quite often in the past for transgene complementation, there are cases in which the 35S-cDNA cannot complement the mutant phenotype, whereas a genomic clone with its native promoter and other regulatory elements does. Thus, it is wise to use genomic clones to do complementation.

Acknowledgments

The authors thank Yu Ti Cheng for help with Table 1, and Sandra Goritschnig and Kristoffer Palma for careful reading of the chapter.

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8

Yeast Two-Hybrid Approaches to Dissecting the Plant Defense Response

Mawsheng Chern, Todd Richter, and Pamela C. Ronald

Summary

We describe a reliable GAL4-based yeast two-hybrid system for identifying and isolating clones encoding proteins interacting with a protein of interest. This two-hybrid system gives extremely low background and few false-positive clones, making it ideal for library screening purposes. We have successfully used it not only to isolate *Arabidopsis* NPR1-interactors from rice but also to pull out the rice NPR1 ortholog using one of the interactors as bait.

Key Words: Yeast two-hybrid; GAL4; NPR1; defense.

1. Introduction

The yeast two-hybrid approach has been widely used for library screening to identify and isolate genes encoding proteins that interact with a favorite protein. It has the capability of identifying and isolating desired clones in a relatively short time by allowing one to easily screen through tens of millions of clones. There are many two-hybrid systems available. Unfortunately, many of them may not be reliable; unacceptable backgrounds and troublesome false-positive results often are seen with many two-hybrid systems. Here, we describe a reliable GAL4-based yeast two-hybrid system that includes a bait plasmid vector derived from plasmid pPC86 ***(1)***, which is based on a *CEN/ARS* DNA replication system rather than the 2 μ replication origin (*see* **Note 1**). This two-hybrid system is compatible with most two-hybrid libraries and gives extremely low background and few false-positive clones, making it ideal for library screening purposes (*see* **Note 2**). We have used it not only to isolate *Arabidopsis* NPR1-interactors from rice but also to pull out the rice ortholog of NPR1 by using one of the NPR1-interactors as bait to back-screen the rice

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

library *(2)*. The unique elements contributing to the successful use of this system are discussed.

2. Materials

1. Plasmid vector pMC86: The construction of this plasmid bas been described previously *(3)*.
2. Yeast strain HF7c.
3. YPD agar plates and liquid medium.
4. Synthetic dropout (SD) media. SD-Trp, SD-Trp-Leu, and SD-Trp-Leu-His + 10 m*M* 3-AT plates. SD-Trp liquid medium.
5. 1X TE/LiAc. Prepare from 10X LiAc (1N) and 10X TE (0.1 *M* Tris-HCl, 10 m*M* ethylene diamine tetraacetic acid, pH 7.5). For storage of yeast competent cells, add sterile glycerol to approx 25%.
6. Polyethylene glycol (PEG)/LiAc solution containing 40% PEG. Make fresh by mixing 1 v of 10X TE, 1 v of 10X LiAc, and 8 v of 50% PEG stock. Do not subject 50% PEG to prolonged autoclaving; autoclave briefly or filtrate.
7. Denatured, sheared herring testes carrier DNA (10 mg/mL).
8. Dimethyl sulfoxide.
9. Z buffer: $Na_2HPO_4{\cdot}7H_2O$ (16.1 g/L), $NaH_2PO_4{\cdot}H_2O$ (5.5 g/L), KCl (0.75 g/L), $MgSO_4{\cdot}7H_2O$ (0.246 g/L), pH 7.0 and autoclave.
10. X-gal/Z buffer: Mix 66.8 µL X-gal stock solution (20 mg/mL in dimethylformamide) and 10.8 µL of β-mercaptoethanol with 4 mL of Z buffer.
11. Yeast lysis solution: 2% Triton X-100, 100 m*M* NaCl, 1% sodium dodecyl sulfate, 1 m*M* ethylene diamine tetraacetic acid, and 10 m*M* Tris-HCl, pH 8.0.

3. Methods

The following protocols are derived mainly from Clontech yeast protocols.

3.1. Preparation of the Bait Plasmid and Yeast Competent Cells

1. Construct bait plasmid coding for a fusion protein of the GAL4 DNA binding domain and your favorite protein (*see* **Note 3**) by using the multiple cloning sites available in pMC86. The pMC86 plasmid, carrying the GAL4 DNA binding domain and the *Trp1* selection marker, is compatible with vectors with the *Leu2* selection, such as pAD-GAL4 from Stratagene, for library construction. For library vectors that carry the *Trp1* selection, the pPC97 plasmid can be used for bait construction instead.
2. Inoculate a fresh colony of yeast HF7c in 3 mL of YPD liquid medium in the morning and grow the culture at 30°C with vigorous shaking. Inoculate 50 mL of YPD liquid medium with this 3 mL of HF7c culture at the end of the day and grow overnight.
3. Centrifuge yeast cells the next morning at 3000*g* at room temperature for 5 min, remove the supernatant, resuspend cells in 30 mL of sterile water, and spin down again. To prepare competent cells for storage, resuspend the yeast cells in sterile 1X TE/LiAc containing 25% glycerol. The competent cells can be stored

at –80°C for at least 1 yr; however, transformation efficiency will gradually decrease. As an alternative, the yeast transformation kit from Zymo Research (Orange, CA) works fairly well.

4. Transform HF7c competent cells with the bait plasmid: Mix 1 μL of pMC86-derived bait plasmid DNA (approx 0.1 μg) and 5 μL of denatured herring testes carrier DNA (10 mg/mL) with 50 μL of the competent yeast cells. Add 300 μL of PEG/LiAc solution to the cells, mix, and incubate at 30°C with shaking for 30 to 60 min. Spread the mixture directly on a SD-Trp plate. Yeast colonies will appear in 2 to 3 d. Streak out individual colonies and make glycerol stocks in YPD medium plus 25% glycerol.

3.2. Library-Scale Transformation With Library DNA (Prey Plasmid)

1. Purify library DNA by using large-scale plasmid purification columns, such as Qiagen Maxi plasmid columns. Normally, at least several hundred micrograms of library DNA is needed.
2. On the morning of day 0, inoculate 3 mL of SD-Trp medium with several fresh colonies of HF7c containing the bait and grow at 30°C with vigorous shaking. At the end of the day, inoculate 100 mL of SD-Trp medium with this 3-mL seed culture and grow overnight (*see* **Notes 4** and **5**). The culture should almost reach stationary phase at the end of day one. Inoculate 1000 mL of SD-Trp medium with the whole 100-mL culture and grow overnight. In the morning of day 2, the culture should reach the late log phase. Spin down the yeast cells at 5000*g* for 5 min at room temperature. Remove the supernatant. Resuspend cells in 500 mL of sterile water and spin down cells again.
3. Resuspend the cells in 8 mL of 1X TE/LiAc. Mix 2 mL of denatured herring testes carrier DNA (10 mg/mL) and 100 to 500 μg of library DNA (prey plasmid) with the cells. Add this mixture to 60 mL of PEG/LiAc solution. Mix well.
4. Incubate at 30°C for 30 min with shaking.
5. Add 7 mL of dimethyl sulfoxide. Mix well with swirling.
6. Heat shock for 15 min at 42°C with occasional swirling. Chill cells on ice for 1 to 2 min (*see* **Note 5**).
7. Spin at 5000*g* for 5 min at room temperature to pellet cells. Remove the supernatant.
8. Resuspend the cells in 10 mL of 1X TE, pH 7.5.
9. Plate out cells on SD-Trp-Leu-His medium containing 10 m*M* 3-AT. This procedure requires approx 50 150 × 15-mm plates. Also plate out a small aliquot of cells on SD-Trp-Leu medium to estimate the total amount of yeast transformants. Incubate the plates at 30°C for up to 2 wk. Seal plates after a few days of incubation to slow down plate drying.
10. This protocol typically yields several to 20 million yeast transformants that grow on SD-Trp-Leu medium.

3.3. Perform β-Galactosidase Filter Assay to Select Positive Clones

1. Yeast colonies of putative interactors would start to appear in 5 d. Some yeast colonies may not show up until 2 wk after transformation.

2. Streak good colonies to new SD-Trp-Leu-His plates containing 10 m*M* 3-AT. The cells should grow into patches in 2 to 3 d.
3. Scrape up half of the cell mass of each clone from plate and patch on a sterile 3-mm filter circle with sterile toothpicks. Place the filter circle on a SD-Trp-Leu plate and incubate at 30°C overnight.
4. Lift the filter circle and air-dry it. Dip it in liquid N_2 for 10 s to permeabilize yeast cells. Thaw the filter at room temperature for 1 to 2 min.
5. At this time, add approx 1.9 mL of X-gal/Z buffer solution to a filter circle in a plate (100 × 15 mm) to prepare an X-gal-saturated filter circle.
6. Overlay the cells/filter on the X-gal-saturated filter in the plate.
7. Incubate at 30°C or room temperature until blue colors develop. This may take an hour to overnight incubation depending on the strength of interaction.

3.4. Isolation of Plasmid DNA From Yeast and Retransformation of Yeast Cells for Confirmation of Positive Interaction

1. Scrape up yeast cells from plates and resuspend them in 200 μL yeast lysis solution. Add 200 μL of phenol/chloroform and 200 mg of acid-washed glass beads.
2. Vortex for 2 min to break cells. Spin at 14,000 rpm for 5 min at room temperature.
3. Transfer the supernatant to a clean microcentrifuge tube. Add two volumes of ethanol and 1/10 volume of 3 *M* NaOAc to precipitate the DNA.
4. Spin down DNA and rinse the pellet with 70% ethanol. Dry the pellet.
5. Resuspend the DNA pellet in 20 μL of Tris-HCl or TE buffer, pH 8.0.
6. Transform *Escherichia coli* cells with 1 μL of the DNA by electroporation.
7. Pick two transformed *E. coli* colonies, grow in 2 mL of luria broth medium with carbenicillin or ampicillin, and extract plasmid DNA by miniprep.
8. Cut DNA with enzymes and run on a gel to confirm the presence of the prey plasmid and the insert. Based on the restriction patterns, the isolated clones can often be divided into groups.
9. Transform HF7c yeast cells with the isolated prey plasmid and the bait plasmid simultaneously. Plate out on SD-Trp-Leu medium.
10. Transfer several colonies for each putative clone to a SD-Trp-Leu-His plate containing 10 m*M* 3-AT to test for growth. Also perform β-galactosidase assay to confirm the interaction.

4. Notes

1. The pMC86 plasmid, derived from pPC86 and pPC97 ***(1)***, is based on the *CEN6/ARSH4* replication system, different from the 2-μ replication origin. In contrast to the high copy number of 2-μ-based plasmids, the *CEN6/ARSH4* replication system-based pMC86 has a low copy number. We have noticed that when a 2-μ-based plasmid is used as bait, the number of false-positive clones tends to be higher, possibly as a result of the higher DNA recombination events between the plasmid DNA and the genome. In addition to lowering false-positive clones, this feature of pMC86 also makes easier the recovery of the prey plasmid like pAD-

GAL4, carrying the 2-μ replication origin because most of the plasmid DNA recovered from yeast would be the prey plasmid.

2. The HF7c yeast strain, carrying *Trp1*, *Leu2*, and *His3* selectable markers and the *LacZ* reporter gene, has an extremely low background when plated on SD-Trp-Leu-His medium containing 10 m*M* 3-AT. When streaked directly on plate, HF7c does not require addition of 3-AT to suppress its growth. This feature is critical in library screening. On the contrary, many other commonly used yeast strains, such as PJ69-4A, carry significant leaky *His3* activity and require much higher 3-AT concentrations to suppress their growth, often give high backgrounds during library screening. It is possible to lower the concentration of 3-AT in medium for screening when using HF7c. In general, the level of background is proportional to the cell mass spread on each plate. The more cell mass on each plate, the higher concentration of 3-AT is needed.
3. We have noticed that the larger the protein encoded by the bait, the higher the number of false-positive clones, which may be attributable to the fact that yeast contains in its genome many protein sequences that can serve as a transcription activation domain and that DNA recombination rates are high in yeast. Any recombination event that creates a fusion protein between the bait and a transcription activation domain will generate a false-positive clone. In general, it is a good practice to keep the coding sequence of the bait less than 2 kb to avoid high number of false-positive clones.
4. However, HF7c does carry a disadvantageous feature; it often does not grow very vigorously in the SD-Trp medium and requires more time to grow to the needed cell mass compared to some other strains. This feature may contribute to lower transformation efficiency sometimes.
5. Some bait constructs may result in slower growth of the HF7c cells. This would usually give rise to lower transformation efficiency. To boost transformation efficiency, one can grow the HF7c cells in YPD medium for 2 h after spinning down cells from the 1000 mL SD-Trp culture before proceeding to transformation. After the heat shock treatment, the cells can also be cultured in YPD medium for a few hours before spun down for plating in order to help the yeast cells recover from the treatments.

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9

Use of Rolling-Circle Amplification for Large-Scale Yeast Two-Hybrid Analyses

Xiaodong Ding, Yan Zhang, and Wen-Yuan Song

Summary

Detection of protein–protein interactions on a large-scale has become a major focus of functional genomics after the completion of genome sequencing. The information generated from these studies not only assembles proteins into signaling networks, but also reveals potential functions of uncharacterized proteins when their interacting partners have known functions. We have developed a rolling circle amplification-based yeast two-hybrid scheme that allows one to test reproducibility and specificity of the interactions on a large scale. Using this scheme, technical false-positives from yeast two-hybrid analyses can be efficiently minimized.

Key Words: Protein–protein interactions; yeast two-hybrid; rolling-circle amplification; high throughput; plasmids.

1. Introduction

Proteins often exist by interacting with other proteins to fulfill their physiological role. Such interactions are critical for the stabilization and subcellular localization of many proteins. The protein–protein interactions also regulate enzymatic activities and provide the connectivity and specificity of signaling networks. Yeast two-hybrid analysis is a genetic method of choice for detecting pair-wise protein–protein interactions in a cellular setting ***(1–3)***. Instead of using complex technologies to purify protein complexes and identify interacting partners, yeast two-hybrid analysis manipulates plasmids in yeast to test for interactions of the proteins (also called bait and prey) produced by the plasmids. It has been estimated that more than half of the protein interactions reported in the literature were originally identified by yeast two-hybrid analyses ***(4)***. The viability of this method relies on its low costs, simplicity in manipula-

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

tion, and sensitivity in detection (both stable and transient interactions can be detected by yeast two-hybrid).

Like any technique used for the study of protein–protein interactions, the current yeast two-hybrid procedures have their limitations. A large number of false-positives have been observed in a variety of yeast two-hybrid screenings, particularly high-throughput analyses. These include the artificial interactions resulting from the activation of the yeast two-hybrid reporters in the absence of interacting proteins. This group of false-positive interactions has been categorized as technical false-positives ***(5)***. It has been estimated that as many as 40% of the interactions, obtained from the initial library screen, cannot be confirmed by retransforming the identified prey into fresh yeast cells that contains the original bait ***(5)***. However, examination of the reproducibility of the interactions is often not performed in many high-throughput yeast two-hybrid analyses because plasmid isolation from yeast cultures and subsequent propagation in *Escherichia coli* are extremely labor-intensive and time-consuming when carried out on a large scale.

Rolling-circle amplification (RCA), used by bacteria to replicate circular plasmids or viruses in nature ***(6)***, has been developed as a powerful tool to amplify plasmid DNA in vitro ***(7,8)***. Because of its proofreading and high processive activities, the Phi29 DNA polymerase used in RCA can efficiently amplify plasmids with a broad size range at a high fidelity ***(9–12)***. The simplicity, robustness, and contamination-resistant features make RCA particularly useful for high-throughput assays. We adapted RCA to simplify yeast two-hybrid procedures ***(12–14)***. The entire bait and prey plasmids can be equally amplified from single-yeast colonies or isolated plasmids in a 96-well format. The amplified, linear concatemeric DNA is sufficient and suitable for a variety of molecular analyses including restriction digestion, DNA sequencing, yeast transformation, and even bacterial transformation ***(13,14)***. When retransformed into yeast, the bait plasmid can be excluded from the prey using distinct counterselection methods, which allows for the examination of specificity and reproducibility of the interactions. By using the RCA-based yeast two-hybrid scheme, the interactors found in the initial library screen can be verified in subsequent one-on-one-based assays.

2. Materials

2.1. Yeast Strains

1. CG1945 (*MAT***a** *ura3-52 his3-200 lys2-801 trp1-901 ade2-101 leu2-3,112 gal4-542 gal80-538 LYS2*::*GAL1-HIS3 cyh*^{r}*2 URA3*::[*GAL4* 17-mers]$_3$-*CYC1-lacZ*).
2. Y187 (*MAT*α, *ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4*Δ, *gal80*Δ, *met*$^-$, *URA3*:: *GAL1*$_{UAS}$ - *GAL1*$_{TATA}$ - *lacZ MEL1*).

3. MaV203 (*MAT*α *leu2-3,112 trp1-901 his3200*Δ *ade2-101 cyh2*^{r} *can1*^{r} *gal4*Δ *gal80*Δ *GAL1*::*lacZ HIS3*$_{UASGAL1}$::*HIS3@LYS2 SPAL10*$_{UASGAL1}$::*URA3*).

2.2. Yeast Media

1. Yeast peptone dextrose (YPD; 1.0 L): To 950 mL of H_2O, add 10 g of bacto-yeast extract, 20 g of bacto-peptone, 18 g of agar (for plate only). Adjust pH to 5.8, autoclave, and cool to approx 55°C. Then add dextrose (glucose) to 2% (50 mL of a sterile 40% stock solution).
2. Yeast complete medium (YCM; 1.0 L): To 950 mL of H_2O, add 10 g of bacto-yeast extract, 10 g of bacto-peptone, 18 g of agar (for plate only). Adjust pH to 3.5 (liquid) or 4.5 (agar plate), autoclave, and cool to approx 55°C. Then add dextrose (glucose) to 2% (50 mL of a sterile 40% stock solution).
3. Synthetic dropout (SD; 1.0 L): To 950 mL of H_2O, add 6.7 g of Difco yeast nitrogen base without amino acids (Difco; cat. no. 0919-15-3), 20 g of agar (for plate only) and one of the following amino acid supplements:
   a. SD/-Trp: 0.74 g of -Trp DO supplement (BD Biosciences; cat. no. 630413).
   b. SD/-Leu: 0.7 g of -Leu DO supplement (BD Biosciences; cat. no. 630414);
   c. SD/-Trp-Leu: 0.64 g of -Trp-Leu DO supplement (BD Biosciences; cat. no. 630417).
   d. SD/-Trp-Leu-His: 0.62 g of -Trp-Leu-His DO supplement (BD Biosciences; cat. no. 630419).
   e. SD/-Leu-Ura-His: 0.65 g of -Leu-Ura-His DO supplement (BD Biosciences; cat. no. 8614-1).

   Adjust pH to 5.8, autoclave, and cool to approx 55°C, and then add dextrose (glucose) to 2% (50 mL of a sterile 40% stock solution).
4. SD/-Leu-Ura+Trp(L): To 950 mL of H_2O, add 6.7 g of Difco yeast nitrogen base without amino acids, 0.65 g of -Leu-Ura-Trp DO supplement (BD Biosciences cat. no: 630426), 0.1 mg of tryptophan (Sigma, cat. no. T-0254), and 20 g of agar (for plate only). Adjust pH to 5.8, autoclave, and cool to approx 55°C, and then add dextrose (glucose) to 2% (50 mL of a sterile 40% stock solution).

2.3. Solutions

1. All the solutions are prepared using double-distilled H_2O.
2. 40% Dextrose, autoclaved or filter-sterilized (avoid prolonged or repeated autoclaving).
3. 1 *M* 3-Amino-1,2,4-triazole (3-AT; Sigma, cat. no. A-8056), filter-sterilized.
4. 1 mg/mL Cycloheximide (Sigma, cat. no. C-6255), filter-sterilized.
5. 0.5 g/mL FAA (2-amino-5-fluorobenzoic acid; Fluka, cat. no. 07973) in absolute ethanol.
6. 10 mg/mL Herring testes carrier DNA (single-stranded DNA [ssDNA]; Sigma, cat. no. D-1626).
7. 50% Polyethylene glycol (PEG) 4000 (average mol. wt. = 3350; Sigma, cat. no: P-3640), filter-sterilized.

8. 10X TE buffer: 0.1 *M* Tris-HCl, 10 m*M* ethylene diamine tetraacetic aicd, pH 7.5. Autoclave. 1X TE buffer (TE solution) is prepared by diluting the 10X stock.
9. 10X LiAc: 1 *M* lithium acetate (Sigma, cat. no: L-6883). Adjust to pH 7.5 with dilute acetic acid and autoclave.
10. PEG/LiAc solution: Mix 8 mL of 50% PEG 4000, 1 mL of 10X TE, and 1 mL of 10X LiAc. Prepare just before use.
11. Dimethyl sulfoxide (DMSO; Sigma, cat. no. D-8779).
12. 1 *M* Sorbitol (Fisher, cat. no. BP439-500).
13. 50% Glycerol (Fisher, cat. no. G153-1).

2.4. Equipment and Supplies

1. SmartSpec3000 (Bio-Rad).
2. Centrifuges 5417C and 5810R (Eppendorf).
3. Isotemp 110 waterboth (Fisher).
4. Bioassay dishes (NUNC).
5. Analytical funnels (Fisher).
6. PVC vacuum manifolds (Fisher).
7. HydroTech vacuum pump (Bio-Rad).
8. 47-mm Water membrane (pore size = 0.45 μm; Fisher).
9 96 Deepwell plates (Fisher).
10. 96-Well amplification plates with chimney (NUNC).
11. 96-Hydra microdispenser (Robbins Scientific Corporation).
12. 96-Pin replicators (V&P Scientific).
13. Single-well Omnitrays (NUNC).
14. PTC-200 Peltier Thermo Cycler (Bio-Rad).
15. TempliPhi™500 Amplification kit (Amersham Biosciences).

3. Methods

3.1. Transformation of Bait Constructs Into Yeast (Small-Scale Yeast Transformation)

1. We have constructed a pair of gateway compatible bait vectors (pXDGATcy86 and pXDGATU86) (**Fig. 1**) for RCA-based yeast two-hybrid analysis (*see* **Note 1**). To transform bait constructs into CG1945, streak a small portion of the frozen yeast stock of CG1945 onto a freshly prepared YPD agar plate. Incubate at 30°C for 4 to 5 d.

Fig. 1. Schematic drawing of the pXDGATcy86 **(A)** and pXDGATU86 **(B)** vectors for initial yeast two-hybrid screening and subsequent verification of candidate interactors. The gateway conversation cassette (*att*R1-C[R]-ccdB-*att*R2) inserted between *Sal*I (2) and *Eco*RI (1724) is from Invitrogen. The recombination sites *att*R1 and *att*R2 in this cassette are underlined. T-ADH, yeast alcohol *dehydrogenase* gene transcription terminator; TRP1, *phosphoribosylanthranilate isomerase* gene; ARS4/CEN6, for replication and low copy-number maintenance in yeast; Amp (R), ampicillin resistance; Cm(R), chloramphenicol resistance; ColE1 ori, for replication in *E. coli*;

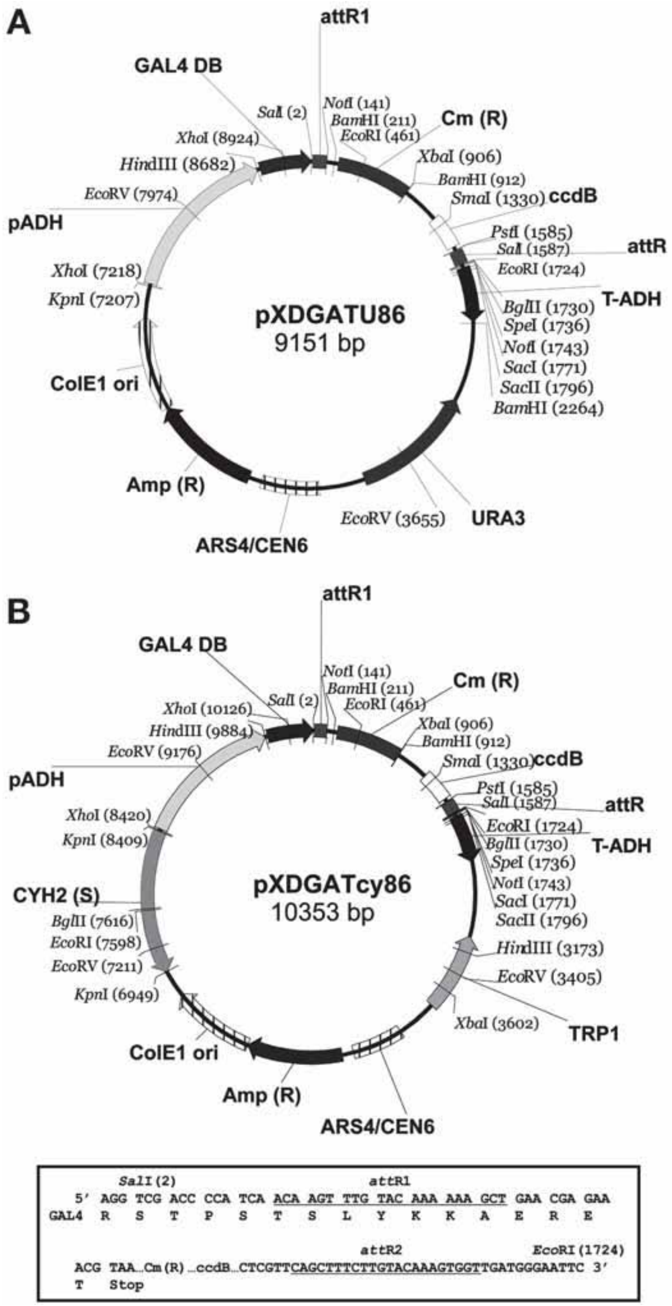

Fig. 1. (*continued from opposite page*) CYR2 (S), cyclohexamide sensitivity; pADH, yeast alcohol *dehydrogenase* gene promoter; GAL4 DB, GAL4 DNA binding domain; URA3, orotidine *5′-phosphatedecarboxylase* gene.

2. Pick two to three colonies (3 mm in diameter) and transfer into 1 mL of freshly prepared YPD liquid medium in a 1.5-mL microfuge tube. Vortex to completely disperse the cells and then transfer into 50 mL of YPD.
3. Incubate at 30°C for approx 18 h with shaking at 250 rpm. Dilute the culture to 300 mL of YPD to make $OD_{600} = 0.2$. Continue to incubate until $OD_{600} = 0.8$.
4. Harvest the cells by centrifugation at 1700*g* for 5 min at room temperature. Wash the cells by resuspending the pellet in 50 mL of H_2O and recentrifugation at 1700*g* for 5 min at room temperature. Discard the supernatant.
5. Resuspend the cell pellet in 1.5 mL of freshly prepared 1X LiAc/TE buffer.
6. Add 10 μL of denatured ssDNA (10 mg/mL), 0.1 μg of pXDGATcy86 or pXDGATU86 derived bait construct, and 0.1 mL of yeast competent cells prepared previously to a 1.5-mL microfuge tube and mix well.
7. Add 0.6 mL of PEG/LiAc solution into the cell mixture and vortex for at least 1 min.
8. Incubate at 30°C for 30 min with shaking at 200 rpm.
9. Add 70 μL of DMSO to a final concentration of 10% and mix gently by inversion.
10. Heat shock for 15 min in a 42°C water bath. Chill the cells on ice.
11. Pellet the cells by centrifugation at 6800*g* for 10 s. Discard the supernatant. Resuspend the cell pellet into 100 μL of TE.
12. Plate the cells on SD/-Trp medium for pXDGATcy86 or SD/-Ura medium for pXDGATU86.
13. Incubate at 30°C for 4 to 5 d.
14. Collect the cells to make glycerol stocks. Store at –80°C for future use.
15. Streak the transformants onto SD/-Trp and SD/-Trp-His media, respectively, to test autoactivation of the bait constructs (*see* **Note 2**).

3.2. Transformation of a Complementary DNA Library Into Yeast (Library-Scale Yeast Transformation)

1. Streak a small portion of the frozen yeast stock of Y187 onto a freshly-prepared YPD agar plate. Incubate at 30°C for 4 to 5 d.
2. Pick two to three colonies (3 mm in diameter) and inoculate into 1 mL of freshly prepared YPD liquid medium in a 1.5 mL microfuge tube. Vortex to completely disperse the cells and then transfer into 50 mL of YPD.
3. Incubate at 30°C for approx 18 h with shaking at 250 rpm. Dilute the culture to 1 L of YPD to make $OD_{600} = 0.2$. Continue to incubate until $OD_{600} = 0.8$.
4. Harvest the cells by centrifugation at 1700*g* for 10 min at room temperature. Wash the cells by resuspending the pellet in 500 mL of H_2O and recentrifugation at 1700*g* for 10 min at room temperature. Discard the supernatant.
5. Resuspend the cell pellet in 8 mL of freshly prepared 1X LiAc/TE buffer.
6. Add 2 mL of denatured ssDNA (10 mg/mL), 400 μg of library DNA (*see* **Note 3**), and 8 mL of yeast competent cells prepared previously into a 50-mL tube and mix well.
7. Combine the aformentioned mixture with 20 mL of 50% PEG in a flask (250 mL) and vortex for at least 1 min.
8. Incubate at 30°C for 30 min with shaking at 200 rpm.

9. Add DMSO to a final concentration of 10% and mix gently by inversion.
10. Heat shock for 15 min in a 42°C water bath. Chill the cells on ice.
11. Pellet the cells by centrifugation at 2700*g* for 10 min. Discard the supernatant. Resuspend the cell pellet into 15 mL of TE solution.
12. Spread the cells onto SD/-Leu medium in about 40 Bioassay dishes (22×22 cm^2). To determine the transformation efficiency, spread 0.2 µL, 0.5 µL, and 1 µL onto the same medium in 100-mm Petri dishes. Incubate at 30°C for 3 d. More than 10 million total transformants are expected for subsequent library screenings.
13. Harvest the cells with 200 mL of 1 *M* sorbitol and briefly disperse the cell suspension at setting 5 for 10 s with an ultrasonic cell disruptor (Microsonix).
14. Mix the cell suspension with an equal volume of 50% glycerol and aliquot 1.2 mL of cells into 1.5-mL tubes.
15. Spread 100 µL, 200 µL, and 500 µL of the diluted cells (10^5- to 10^6-fold) onto SD/-Leu plates. Incubate at 30°C for 3 to 4 d.
16. Wrap the freezer boxes containing the aliquoted cells with five layers of paper towels and store at –80°C.
17. Thaw a tube of frozen cells and determine the viable cells after the freeze/thaw cycle as described in **step 15**.
18. Viability = (cell number after frozen × unit/vol)/(cell number before frozen × unit/vol)%. The expected viability is 40 to 45%.

3.3. Screening of a Complementary DNA Library

1. To screen the complementary DNA (cDNA) library, streak the CG1945 cells carrying the pXDGATcy86 derived bait constructs (stored at –80°C) onto freshly prepared SD/-Trp medium and incubate at 30°C for 4 to 5 d (*see* **Note 4**).
2. Pick two to three (2 mm in diameter) colonies and inoculate into 2 to 3 mL of SD/-Trp liquid medium and shake at 30°C (250 rpm) for approx 18 h.
3. Dilute the culture to $OD_{600} = 0.2$ in 20 mL of SD/-Trp medium and continue to shake for 4 to 5 h until $OD_{600} = 0.8$.
4. Thaw α-mating type cells containing the cDNA library (stored at –80°C as described above) at room temperature for 10 to 15 min.
5. Mix 1.6×10^8 (approx 8 mL) cells containing the bait construct with the thawed cDNA library cells (7×10^7 viable cells) to make the 2.5:1 (bait:library) cell ratio.
6. Centrifuge at 1700*g* for 2 mins and discard the supernatant.
7. Resuspend the cells in 2.3 mL of YCM (pH 3.5) to make a cell density of 10^8 cells/mL.
8. Shake at 220 rpm for 105 min at 30°C.
9. Dilute the cells 100-fold by adding H_2O and vortex at maximum speed for 1 min to disperse the cells.
10. Harvest the cells onto a 47-mm water membrane (pore size = 0.45 µm) using vacuum filtration (**Fig. 2**; *see* **Note 5**).
11. Transfer the membrane (cell side up) onto YCM medium (pH 4.5) and incubate for 4.5 h at 30°C (*see* **Note 6**).
12. The zygotes can be observed under a microscope (pick cells with a tip, resuspend into 100 µL of H_2O, and spread onto a glass slide).

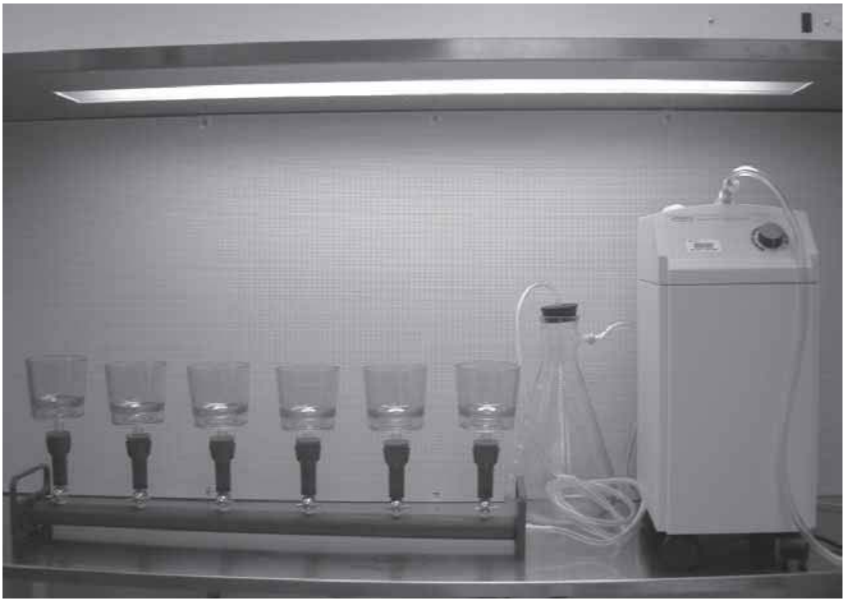

Fig. 2. Six-channel filtration system. Yeast cells in liquid medium are collected on the 47-mm water membranes contained in the funnels by the vacuum drawn from a pump (right).

13. Transfer the membrane into 10 mL of 1 *M* sorbitol solution and vortex vigorously for 1 min to wash the cells off the membrane.
14. Pellet the cells by centrifugation at 1700*g* for 2 to 3 min.
15. Resuspend the cells into 2 mL of TE solution by vortexing for 1 min.
16. Spread the cells onto SD/-Trp-Leu-His + 2 m*M* 3-AT in four Bioassay dishes (*see* **Note 7**).
17. Spread 0.1 µL and 0.2 µL of the cell suspension in **step 15** onto selective medium (SD/-Leu, SD/-Trp, and SD/-Trp-Leu) to determine the mating efficiency: Mating efficiency = Total number of colonies on SD/-Trp-Leu/the sum of total number of colonies on SD/-Trp and SD/-Leu.
18. Incubate at 30°C for 6 to 10 d.
19. Pick the colonies growing on the SD/-Trp-Leu-His + 2 m*M* 3-AT medium and inoculate into 1 mL of the SD/-Trp-Leu-His medium contained in a 96-deepwell plate (master plate) with a glass bead in each well. Incubate at 30°C with shaking at 250 rpm for 2 to 3 d.
20. Transfer 200 µL of cells from each well, of the master plate, into two fresh 96-well microplates with chimney, respectively, by using the 96-Hydra microdispenser. Pellet the cells by centrifugation at 1700*g* for 2 min and discard the supernatant. Add 600 µL of 50% glycerol into each well of the master plate. Store the three plates at –80°C.

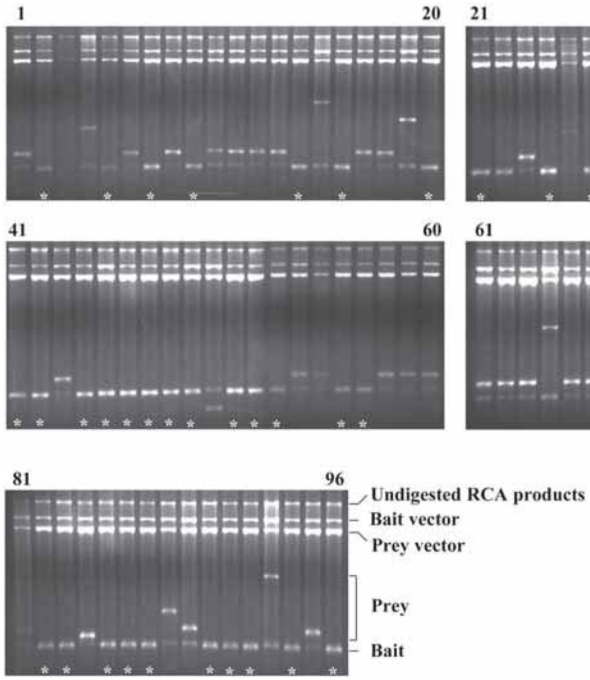

Fig. 3. High-throughput amplification of both bait and prey from the yeast cells using rolling-circle amplification. Cell lysates from single-yeast colonies were used as templates for the amplification. The amplified DNA was digested with *Sal*I and *Not*I. The samples were resolved by agarose gel electrophoresis. The prey samples containing inserts identical in size to the bait are indicated by asterisks.

3.4. Amplification of Bait-and-Prey Plasmids by RCA

1. To prepare the templates for RCA, thaw the cells contained in one microplate as described in **Subheading 3.3.**, **step 20** at room temperature. Resuspend the cells into 50 μL of TE solution containing 0.5 μL of zymolase (5 U/μL). Mix well by gently vortexing and incubate at 30°C for 1 h (*see* **Note 8**).
2. Transfer 5 μL of 10X diluted cell lysate into to a new regular 96-well microplate. Heat the plate on a thermocycler at 96°C for 3 min. Chill on ice/water bath for 10 min.
3. RCA is performed using the TempliPhi100 Amplification kit (Amersham Biosciences). Add 0.2 μL of the premixed enzyme mixture (Phi29 DNA polymerase) and 5 μL of reaction buffer (Amersham Biosciences) into each well of the above plate. Briefly vortex and centrifuge.
4. Incubate at 30°C for 20 to 30 h.
5. The amplified DNA can be analyzed by restriction digestion followed by gel electrophoresis (**Fig. 3**). Alternatively, a pipettor tip can be used to check the viscosity, as the RCA amplified products are concatemeric DNA.

6. Add 15 μL of H_2O to dilute the amplified DNA. Transfer 10 μL into two new plates, respectively. One plate is subjected to DNA sequencing, whereas the other one is used for yeast retransformation as described herein.

3.5. Retransformation of RCA DNA Into Yeast

1. Streak the MaV203 strain carrying the pXDGATU86 derived verification bait onto a freshly prepared SD/-Ura +5 μg/mL cycloheximide medium. Incubate at 30°C for 6 to 7 d.
2. Pick two to three (2 mm in diameter) colonies and inoculate into 5 mL of SD/-Ura medium and incubate at 30°C for approx 18 h with shaking (250 rpm).
3. Dilute 1 mL of the cell culture into 50 mL of SD/-Ura (for transformation of 96 samples).
4. Incubate at 30°C for 6 to 8 h until $OD_{600} = 0.8$.
5. Harvest the cells by centrifugation at 1700*g* for 5 min at room temperature. Wash the cells by resuspending into 50 mL of H_2O and recentrifuge at 1700*g* for 5 min at room temperature.
6. Resuspend the cells into 1.0 mL of 1X LiAc/TE buffer.
7. Add 100 μL of denatured ssDNA (10 mg/mL) to the cell suspension and mix well. Aliquot 10 μL of the cell mixture into each well of the 96-well plate containing the RCA amplified DNA in **Subheading 3.4.6.**
8. Incubate at room temperature for 15 min.
9. Add 50 μL of PEG/LiAc solution containing 10% DMSO to each well. Gently and thoroughly mix.
10. Incubate at 30°C for 30 min and heat shock in a 42°C water bath for 30 min.
11. Transfer 10 μL of the cells using a 96-pin replicator (V&P Scientific, INC) onto a SD/-Leu-Ura+10 μg/mL cycloheximide medium.
12. Dry the plate in a clean hood for 10 to 20 min and incubate at 30°C for 6 to 7 d.
13. Replicate the colonies growing from the above plate onto SD/-Leu-Ura+Trp(L) + 0.5 g/L FAA medium (*see* **Note 9**).
14. Incubate at 30°C for 2 to 3 d.
15. Replicate the cells on SD/-Leu-Ura medium and incubate at 30°C for 3 d.
16. Replicate the colonies growing on the above plate onto SD/-Leu-Ura-His + 40 m*M* 3-AT medium and incubate at 30°C for 6 to 7 d (**Fig. 4**; *see* **Note 10**).

4. Notes

1. The pXDGATcy86 vector, derived from the pPC86 and pPC97 plasmids ***(12)***, is designated for initial library screen. The plamid contains all the features of a bait vector including the sequences coding for the GAL4 DNA binding domain (GAL4-DB) followed by a gateway cassette, the *TRP1* marker for selecting the presence of this plasmid in yeast cells, and the $CYH2^S$ marker. The gateway cassette facilitates the rapid cloning of a gene of interest into this vector using the gateway reactions, whereas the *TRP1* and $CYH2^S$ markers allow for the elimination of pXDGATcy86 derived constructs by the FAA (2-amino-5-fluorobenzoic

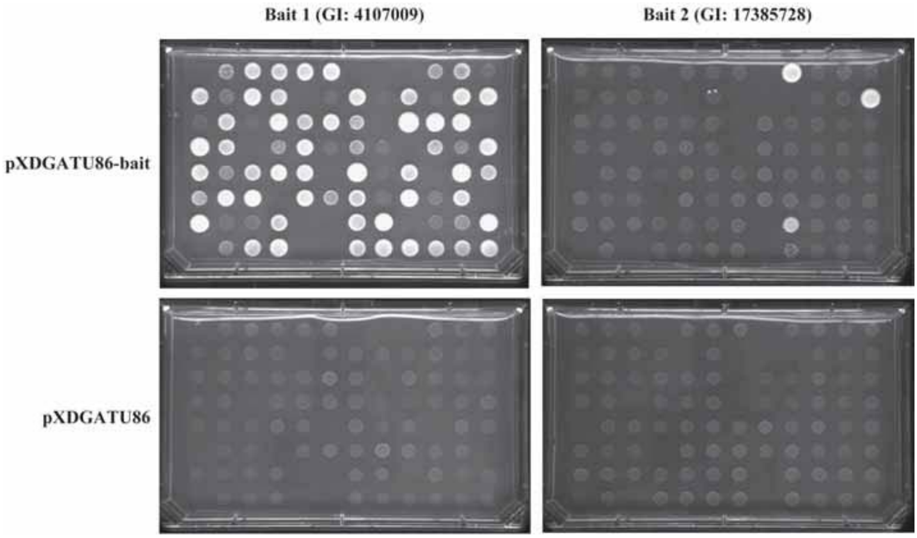

Fig. 4. Verification of candidate interactors by yeast retransformation. Yeast cells, containing the constructs indicated on the left and top, were transformed with candidate interactors and grown on the selective medium (SD/-Leu-Trp-His+ 40 m*M* 3-AT). Colonies capable of growing on the selective medium indicate activation of the reporter gene *His3*.

acid) and/or the cycloheximide counterselection in the RCA products after yeast re-transformation ***(12,15)***.

The pXDGATU86 bait vector is developed for the verification of the identified interactions. Unlike pXDGATcy86, this vector does not contain the above counterselection markers. The *URA3* gene can be used for selecting the presence of this plasmid in yeast cells. Before library screening, the gene of interest is in-frame fused with the GAL4-DB domain in the two bait vectors, respectively. We transform the pXDGATcy86 derived bait constructs into the yeast strain CG1945 for two-hybrid library screening. The **a**-mating type strain (e.g., CG1945) can be mated with an α-mating type strain (e.g., Y187) carrying a cDNA library. In addition, the wild-type CG1945 is resistant to cycloheximide, therefore can be subjected to cycloheximide counterselection when transformed with the pXDGATcy86 derived bait.

To transform a bait vector into CG1945, a number of procedures, for example, Walhout et al. ***(16)***, are suitable. The protocol described here is modified from the user manual of the MATCHMAKER GAL4 Two-Hybrid system (Clontech).

2. Certain gene products can activate the transcription of reporter genes in a prey-independent manner. These bait constructs are not suitable for yeast two-hybrid screening. To test the autoactivation capability of bait, the frozen cells carrying

the pXDGATcy86 derived construct are streaked onto SD/-Trp and SD/-Trp-His media, respectively. If a construct supports the growth of yeast cells these two types of media after incubation at 30°C for 5 to 6 d, the bait autoactivates the *HIS3* reporter.

3. The cDNA libraries are constructed using the HybriZAP-2.1 Two-Hybrid system (Stratagene) by following the manufacture's instructions. The pAD-GAL4-2.1 vector, containing the *LEU2* marker, is compatible with the pXDGATcy86 and pXDGATU86 vectors. Other cDNA libraries constructed using a similar vector may also be compatible to the bait vectors.
4. The screening procedure is modified from Soellick and Uhrig ***(17)***. With the standard laboratory equipment and our multichannel filtration system, we can conduct 15 to 20 screenings simultaneously.
5. We have assembled a six-channel filtration system for rapid harvest of yeast cells onto membranes for mating. The cells, resuspended in 250 mL of H_2O, are vortexed at maximum speed, poured into a funnel containing a 47-mm water membrane (pore size = 0.45 μm) and collected onto the membrane after a vacuum is drawn by a pump. Six samples can be processed simultaneously. To further increase the throughput, additional six-channel filtration apparatus can be added to the system.
6. When transferring the 47-mm water membrane containing the yeast cells onto the solid YCM (pH 4.5) medium for mating, do not allow any bubbles to occur between the membrane and the medium.
7. Do not apply too many cells onto the selection medium (about 3–4 million diploid zygotes/Bioassay dish). The screening stringencies, adjusted by the concentration of 3-AT in the media, vary greatly with different yeast strains.
8. Both cell lysates and isolated plasmids can be used as templates to amplify bait and prey plasmids. Compared with cell lysates, higher yields and reproducibility of amplification can be achieved by using the isolated plasmids. We use the Yeast Plasmid Miniprep kit (Zymo Research, cat. no. D2001) to isolate plasmid DNA from yeast as the template for RCA. In brief, add 30 μL of buffer 1 containing 0.2 μL of zymolase into each well by a 12-channel pipettor. Mix well and incubate at 30°C for 1 h. Add 30 μL of buffer 2 to lyse the cells and add 30 μL of buffer 3. Mix well and centrifuge at 15,000*g* for 10 min. Transfer 100 μL of the supernatant into a new plate and mix with an equal volume of 2-propanol. Centrifuge at 15,000*g* for 20 min. Discard the supernatant.
9. For FAA counterselection, the concentration of tryptophan in the medium must be lowered to 0.1 mg/L. To avoid the carrying over of a clump of yeast cells, do not transfer too many cells onto counterselection medium. The reason for this is to ensure that all of the cells come into contact with the counterselection medium, thus the bait carrying yeast cells can be eliminated.
10. We consider the interactions that cannot be confirmed by yeast retransformation as false-positives. After analyses of more than 60 rice kinases, we found that the false-positive rate varies significantly depending on the baits. **Figure 4** shows the verification results from two rice kinases. For the first kinase, most of the

interactions identified from the initial library screening can be confirmed by retransformation. In contrast, the majority of interactors identified from the second kinase failed to be scored as positives after yeast retransformation.

Acknowledgments

We thank Dr. M. Vidal for providing the yeast strain MaV203, Dr. P. C. Ronald for the precursors of the pXDGATcy86 and pXDGATU86 vectors, and Lisa Nodzon for critical reading of the manuscript. This research was supported by the Florida Agricultural Experiment Station and a grant from the National Science Foundation Plant Genome Research to W-Y S. This work was approved for publication as Journal Series No. R-10865.

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10

Preparative Denaturing Isoelectric Focusing for Enhancing Sensitivity of Proteomic Studies

Antonio Serna-Sanz, Greg Rairdan, and Scott C. Peck

Summary

Substantial evidence implicates important roles for both protein phosphorylation and protein degradation in regulation of plant defense responses. Therefore, interest is growing in applying proteomics techniques to investigate these posttranscriptional changes. We have found, however, that most proteins of interest are not visible on two-dimensional (2D) gels without previous prefractionation. This chapter describes the use of preparative denaturing isoelectric focusing to enrich for proteins of specific isoelectric points before separation by 2D gels. This method significantly increases the sensitivity of 2D gel-based comparisons.

Key Words: Proteomics; 2D gel; preparative isoelectric focusing; phosphoproteomics.

1. Introduction

After recognition of microbial elicitors, plants initiate a number of rapid defense responses. Although it is clear that phosphorylation plays a key role in initiating these responses, little is known about the regulatory proteins involved. Although it is possible to use radioactive pulse-labeling of phosphoproteins in cell culture to identify signaling components ***(1,2)***, we have found that the majority of radioactively labeled proteins cannot be aligned with stained protein spots on two-dimensional (2D) gels using total protein extracts. Signaling proteins are typically low in abundance, and phosphorylation is rarely an event of high stoichiometry. This combination of factors generally means that the target, modified forms of the proteins will be present at exceedingly low levels. Because there is a restriction in the amount of total protein that can be loaded onto 2D gels, simply increasing the load is not an option. Thus, prefractionation of protein samples is essential for meaningful proteomic comparisons. We have found preparative liquid isoelectric focusing (IEF; e.g. Rotofor IEF, Bio-Rad)

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

a powerful and complementary tool for the identification of proteins using 2D gels and mass spectrometry. This procedure prefractionates proteins on the same principles as the first-dimension IEF separation for 2D gels. The end result is that rather than loading 1 mg of total protein on a single isoelectric point (p*I*) unit zoom gel, resulting in the majority of protein focusing outside of the target region, IEF prefractionation allows loads of 1 mg of protein only from the p*I* unit of interest. Thus, this method substantially increases sensitivity and allows identification of even rare proteins.

2. Materials

1. Protein extraction buffer: 100 m*M* HEPES-KOH, pH 7.5, 5% glycerol, 50 m*M* sodium pyrophosphate, 1 m*M* sodium molybdenate, 25 m*M* sodium fluoride, 15 m*M* ethylenebis(oxyethylenenitrilo)tetraacetic acid, 5 m*M* ethylene diamine tetraacetic acid (EDTA), 0.5% polyvinylpyrrolidine, 1% triton (3 m*M* dithiothreitol added on day of use).
2. 100 m*M* Phenylmethyl sulfonyl fluoride (PMSF) in isopropanol (store at 4°C).
3. 10 m*M* Leupeptin (store at –20°C).
4. 10 μ*M* Calyculin A (store at –20°C).
5. Phenol.
6. Back extraction buffer: 100 m*M* Tris-HCl, pH 8.4, 20 m*M* KCl, 10 m*M* EDTA, 0.4% β-mercaptoethanol.
7. 100 m*M* Ammonium acetate in methanol.
8. 80% Acetone, 50 m*M* Tris-HCl, pH 8.0.
9. Sonicating water bath.
10. Low-stringency buffer: 9 *M* urea, 1% Triton X-100, 5% ampholytes, 0.5% DTT.
11. High-stringency buffer: 7 *M* urea, 2 *M* thiourea, 4% CHAPS, 5% ampholytes, 2% DTT.
12. 50% Glycerol.

3. Methods

3.1. Initial Protein Extraction

This protocol is a modification of that previously described by Peck et al. *(2)* and yields proteins of good quality for IEF separation. Attempts to perform IEF with samples of insufficient purity (i.e., directly using a protein extract) will generally result in poor focusing.

1. Immediately before use, add inhibitors to protein extraction buffer (final concentrations of 1 m*M* PMSF, 10 μ*M* leupeptin, and 10 n*M* Calyculin A; *see* **Note 1**). We recommend using approx 2 mL extraction buffer per gram of fresh weight.
2. Centrifuge (10,000*g*, 10 min) to clear cell debris.
3. Transfer the supernatant to a tube containing 1 vol of phenol, vortex, and keep on ice for 5 min.

4. Centrifuge (10,000*g*, 10 min) and discard the aqueous phase. Be careful not to disturb the interface (protein will be in the phenol phase and interface).
5. Add one volume of back extraction buffer, vortex, and centrifuge (10,000*g*, 10 min). Discard the aqueous phase, and repeat the back extraction. **Steps 3** to **5** will help remove both nucleic acids and sugars that would interfere with protein focusing.
6. Add 5 vol of 100 m*M* ammonium acetate in methanol, vortex, and place at –20ºC for 20 min to precipitate proteins from the phenol.
7. Centrifuge (10,000*g*, 10 min) to pellet protein.
8. Wash the pellet with 100 m*M* ammonium acetate in methanol (*see* **Note 2**), using a sonicating water bath to break up the pellet. Centrifuge (10,000*g*, 10 min) and repeat wash.
9. Wash the protein pellet from previous step with 80% acetone buffered with 50 m*M* Tris-HCl, pH 8.0. Again, use a sonicating water bath to break up the pellet. Centrifuge (10,000*g*, 10 min) and repeat wash. Protein can be stored as an acetone suspension indefinitely at –20º C.

3.2. Preparative Isoelectric Focusing

1. After thoroughly mixing the acetone suspension, remove an amount equivalent to 20 to 40 mg of protein to a fresh tube (*see* **Note 3**). Centrifuge (10,000*g*, 10 min), discard supernatant, and allow pellet to air-dry.
2. Resuspend pellet in denaturing IEF buffer (*see* **Note 4**). Determine the volume capacity for the IEF unit (*see* manufacturer's instructions). Prepare the proper amount of chemicals for half this volume to be prepared with double-distilled water and the other half to be prepared with 50% glycerol. The glycerol is necessary to prevent protein precipitation during focusing, but the proteins will dissolve more easily in the glycerol-free buffer.
3. Resupend the pellet in the buffer without glycerol for 1 h with continuous shaking. It may be necessary to assist resuspension by pipetting and/or using the sonicating water bath.
4. Centrifuge (10,000*g*, 10 min) to remove unsolubilized material.
5. Mix the supernatant with the glycerol-containing buffer. The proteins are ready to be loaded in the IEF cell.
6. Follow manufacturer's instructions for assembling the IEF unit, loading the sample, and focusing conditions. (Specifics may vary depending on IEF unit.)
7. After focusing, you will have many (approx 20) fractions representing a range of p*I*s (**Fig. 1**). We have generally found that mixing two to three adjacent fractions does not greatly decrease sensitivity and substantially reduces the number of samples for processing in **step 8**.
8. Fractions need to be back-extracted to remove ampholytes that would interfere with the ampholyte composition of the IEF strip for first dimension separation of 2D gels. Perform **steps 3–9** from **Subheading 3.1.** on each sample.
9. Again, samples can be stored as acetone suspensions until needed.

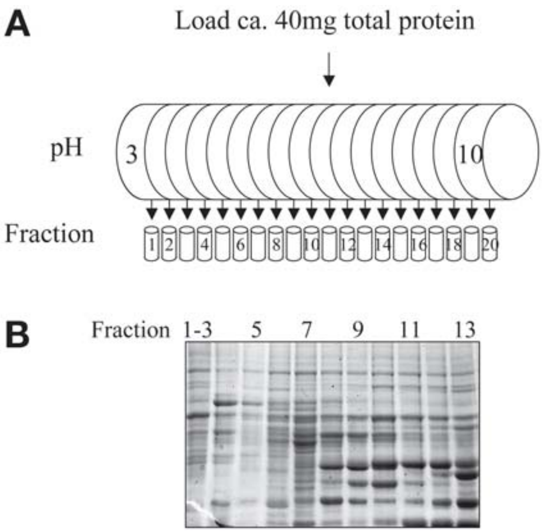

Fig. 1. Fractionation of proteins by preparative isoelectric focusing. **(A)** Protein samples resuspended in a denaturing buffer, mixed with ampholytes, and separated by isoelectric focusing. When focusing is complete, fractions are vacuum collected for further analysis. **(B)** Separation of protein samples (20 μg) from preparative isoelectric focusing fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis show changes in protein patterns between fractions.

10. To use the appropriate first dimension pH gradient for each fraction, we perform an immunoblot analysis using a protein of known p*I* (*see* **Fig. 2**).
11. Alternatively, pH 3.0–10.0 linear-gradient 2D gels can be run with a small amount of sample to determine the corresponding gradients for each fraction.

4. Notes

1. The protein extraction buffer given is for isolation of phosphoproteins. If phosphorylation status is not a consideration, phosphatase inhibitors can be eliminated (i.e., sodium pyrophosphate, sodium molybdenate, sodium fluoride, and Calyculin A). PMSF and Calyculin A are unstable in solution, so they should be added only immediately before protein isolation. The polyvinylpyrrolidine (PVP) is present to bind and remove polyphenolics that might otherwise damage proteins. For older leaves or leaves from difficult species (e.g., solanaceae), it may be necessary to increase the PVP concentration or to include the insoluble polyvinylpolypyrrolidine to remove all polyphenolics. A simple indication is that if the protein extract is turning purple, more PVP or polyvinylpolypyrrolidine should be used.
2. After the first methanol precipitation, the protein pellet tends to be spread along the entire wall of the centrifuge tube. Be sure to use a pipet to remove all protein from the wall of the tube with the first wash. Failure to do so will result in signifi-

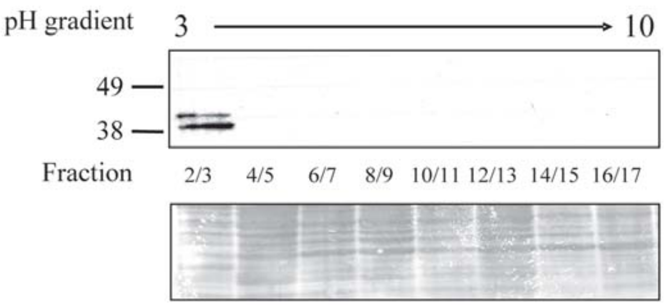

Fig. 2. Using immunoblot analysis to determine isoelectric point (p*I*) cut-off from preparative isoelectric focusing fractions. After preparative isoelectric focusing of proteins from *Lotus japonicus*, sequential fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride. Immunoblots were hybridized with an antibody raised against Phos43 ***(2)***, which has a p*I* of less than 4.3. As seen in the immunoblot analysis, proteins cross-reacting with the Phos43 antibody are found only in the most acidic fractions, indicating that these fractions can be run on expanded p*I* gradients with p*I*s less than 4.5.

cant losses of protein. This step is generally not necessary after subsequent centrifugations.

3. We generally use protein concentrations of approx 0.5 mg/mL. Higher protein concentrations are possible but could result in protein precipitation because of the desalting effect of focusing or the increase of local protein concentration. It may be possible to overcome these problems by increasing ampholyte concentrations (we use 5%, but they theoretically can be raised up to 40%) or by adding more glycerol to the buffer (we use 12.5%, but it may be possible to increase the concentration to 20%).
4. We sometimes find it advantageous to decrease the complexity of the proteome using differential protein extraction from the acetone pellet. The low-stringency buffer resolubilizes a subset of proteins with a general bias toward smaller (<60 kDa) proteins. The remaining pellet can then be resuspended with the high-stringency buffer. In both cases, the amount of urea and/or thiourea is substantial and affects the volume of the solution. Therefore, only add approx 2.9 mL of water for each 5 mL of total volume of low-stringency buffer or 2.5 mL of water for each 5 mL of the high-stringency buffer. Once the chemicals are fully resuspended, adjust the final volume. For the solutions containing glycerol, perform the same procedure but use 50% glycerol instead of water to make the buffers.

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11

Use of Massively Parallel Signature Sequencing to Study Genes Expressed During the Plant Defense Response

Blake C. Meyers, Christian D. Haudenschild, and Kalyan Vemaraju

Summary

Massively parallel signature sequencing is a sequencing-based method that provides quantitative gene expression data for nearly all transcripts in a particular ribonucleic acid sample. Although the sequencing technology is practiced as a service by a California-based company, we have developed methods for the handling and analysis of these data. This chapter describes the steps involved in obtaining data from massively parallel signature sequencing, aligning the signatures to genomic sequence, identifying novel transcripts, and performing quantitative analyses of genes expressed under conditions such as disease treatments.

Key Words: MPSS; massively parallel signature sequencing; SAGE; gene expression; bioinformatics; transcriptional analysis.

1. Introduction

Massively parallel signature sequencing (MPSS) is a novel gene expression technology that has been used extensively in our laboratory for transcriptional analysis of *Arabidopsis* and rice. Like other gene expression technologies such as microarrays and serial analysis of gene expression (SAGE *[1]*), MPSS can be used to monitor the abundance of transcripts in plants faced by challenges, including biotic stress. The characterization of patterns and levels of transcriptional activity under such stress can be used to address specific hypotheses or can be used as the starting point for quantitative analyses of individual genes or gene families. Although MPSS has its limitations, this technology offers certain advantages over other methods for whole-genome expression analyses. Other methods, such as complementary DNA (cDNA) microarrays *(2)*, oligonucleotide microarrays *(3)*, or SAGE may be less expensive and easier to perform in an individual laboratory, but MPSS provides quantitative expression informa-

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

tion and allows the identification of novel transcripts (*4,5*). However, MPSS data have several unique aspects and must be treated in a different way than SAGE data (*6*). Because of this, we have developed novel methods to link plant genomic data with tag- or signature-based expression information. Many of the analysis methods that we describe in this chapter could be used equally well to analyze SAGE data.

MPSS has not been widely used to study plant–pathogen interactions, but the technology is well suited to monitoring transcriptional regulation in the host. In the case of eukaryotic pathogens such as fungal, oomycete, or animal infection, it also may be possible to monitor in parallel the transcriptional events that take place in the pathogen. The limitation to measurements of eukaryotic and not prokaryotic transcription is a result of the use of polyadenylated RNA in MPSS. Because of the depth of sequencing (>1 million transcripts sampled per library), eukaryotic pathogen transcripts may be detected in mixed tissues, although this detection may require high inoculation levels or an advanced stage in the infection. Because the MPSS signatures are generally 17 or 20 nucleotides in length, it is necessary to match these sequences to a sequenced genome or cDNA sequences. Whole- or partial-genome sequences of plants and many of their eukaryotic pathogens are increasingly available, and we believe that the approach of parallel measurements of host and pathogen genomes will be used more widely in the future. In collaboration with the laboratory of Dr. Guo-liang Wang (The Ohio State University), we are currently evaluating host and pathogen transcription in MPSS libraries constructed from rice infected with the rice blast pathogen *Magnaporthe grisea*. Much of the genomic sequence of *Magnaporthe* is now available (*7*), and the rice genome is largely complete; therefore, rice-Magnaporthe represents one of the first plant–pathogen systems in which the host and pathogen can be simultaneously monitored by MPSS.

To facilitate the use and interpretation of MPSS data, we have constructed a customized set of methods, tools, and databases (*8*). Our database and a specialized web interface facilitates public access to gene expression data derived by MPSS. This public web-based resource for *Arabidopsis* is available at http://mpss.udel.edu/at. The MPSS data and *Arabidopsis* genomic sequence and annotation were used as the basis for the development of publicly available analysis and comparison tools. Our web site includes a genome viewer, a set of gene, signature and library analysis pages, an FTP site for retrieval of the data, and a signature extraction tool to allow specific sequence comparisons to the MPSS data. Because the methods that we used for analyzing the MPSS data are critical for the interpretation of the results, we will describe these bioinformatics methods in this chapter.

2. Materials

2.1. Isolation of Total RNA

1. TRIzol Reagent (Invitrogen/Life Technologies).
2. Diethyl pyrocarbonate (DEPC; Sigma).
3. DEPC-treated double-distilled H_2O (ddH_2O): incubate 0.05% DEPC at room temperature for at least 4 h and then autoclave for 45 min at 121°C. Alternatively, it can be purchased from Ambion.
4. 50-mL Polypropylene conical tubes.
5. Mortar and pestle, spatulas.
6. Chloroform.
7. Isopropanol.
8. Absolute ethanol.
9. 75% Ethanol (prepared with RNase-free water and stored at –20°C).
10. Liquid nitrogen.
11. RNase Zap (Invitrogen/Life Technologies).

2.2. Bioinformatics and Analysis of MPSS Data

1. Database server.
2. MySQL. Oracle will facilitate some analyses, but is not necessary.

3. Methods

3.1. Isolation of Total RNA

1. Clean all equipment with soap and rinse with deionized water followed by 100% ethanol. Allow this to dry, and then treat with RNase Zap.
2. Plant tissues should be frozen before RNA extraction, preferably by rapid freezing in liquid nitrogen and storage at –80°C.
3. Chill the mortar by adding a small amount of liquid nitrogen and allowing this to boil away.
4. Homogenize 50 to 100 mg of tissue, transfer to a tube (*see* **Note 1**), and add 1 mL of TRIZOL reagent (*see* **Note 2**), with the sample not exceeding 10% of the volume of TRIZOL (*see* **Note 3**).
5. Incubate the samples for 5 min at room temperature.
6. To remove insoluble material centrifuge at 12,000*g* for 10 min (*see* **Note 4**); the supernatant contains the RNA. Transfer the cleared solution to a fresh tube (*see* **Note 5**).
7. Add 0.2 mL of chloroform for each milliliter of TRIZOL; cap tubes securely and shake vigorously for approx 15 s. Incubate at room temperature for approx 3 min. Centrifuge the samples at 12,000*g* for 15 min at 2 to 8°C. After centrifugation, the mixture separates into a lower phenol–chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization (*see* **Note 6**).

8. Add 0.5 mL of isopropanol and 0.5 mL of (0.8 *M* sodium citrate and 1.2 *M* NaCl) per milliliter of aqueous phase. Mix and leave at room temperature for 5 to 10 min.
9. Spin the precipitate at 12,000*g* for 20 min.
10. Remove the supernatant and wash with 75% EtOH, using 1 mL per milliliter of TRIZOL.
11. Spin sample at 12,000*g* for 15 min, then remove the supernatant; the pellet should appear clear and almost gelatinous.
12. Air dry and resuspend in the appropriate amount of water, using approx 0.5 mL to resuspend to a concentration of approx 1 mg/mL.
13. Typical yield is between 500 and 700 μg of RNA per gram of leaf tissue, with higher yields for tissues with more compact cells such as flowers.

3.2. RNA Quality Analysis and mRNA Purification

1. The total RNA should be analyzed by agarose gel electrophoresis (1% agarose, run at approx 100 V for 30 min) to assess the quality; good-quality RNA should show distinct bands representing ribosomal RNA and one transfer RNA band. If possible, a portion of this RNA may be used for assays of known markers using gel blot analysis, reverse transcription polymerase chain reaction (PCR), microarrays or other methods.
2. Because of the complexity of the methods and the equipment that is required, in practice, MPSS sequencing can only be performed at Solexa, Inc. Therefore, the next step is to send at least 20 μg of purified total RNA to Solexa for sequencing.
3. Solexa verifies the quality of total RNA using an Agilent Bioanalyzer, comparing the ratio of rRNAs and the distribution of RNA sizes.
4. Total RNA that passes initial quality assessments is treated at Solexa with DNase, and polyadenylated RNA is isolated using the Poly(A) Purist messenger RNA (mRNA) purification kit from Ambion (cat. no. 1916). The mRNA is reassessed and quantified using the Agilent Bioanalyzer.

3.3. MPSS Library Construction and Sequencing

1. The first process in MPSS comprises library construction. Through this set of steps, Solexa clones a specific fragment from each mRNA molecule onto a single 5-μm bead; this is performed in parallel for millions of beads. The original process was described in Brenner et al. ***(5)***, and an overview is shown in **Fig. 1**. Double-stranded cDNA is prepared with approx 100 ng of mRNA, using biotinylated oligo-dT for reverse transcription followed by second-strand cDNA synthesis.

Fig. 1. Overview of massively parallel signature sequencing (MPSS) library construction. Poly-A messenger RNA (mRNA) is converted into double-stranded complementary DNA (cDNA) and ultimately cloned onto microbeads for sequencing by MPSS. The 3'-most *Dpn*II fragment of the cDNA is captured using a biotinylated oligo-dT primer. This fragment is further trimmed to a 21 or 22 nucleotide signature that is cloned adjacent to a unique 32-base oligonucleotide "combitag" such that each cDNA signature is linked to one of approx 16.7 million possible combitags. After amplification by polymerase chain reaction (PCR), the combitags are made single

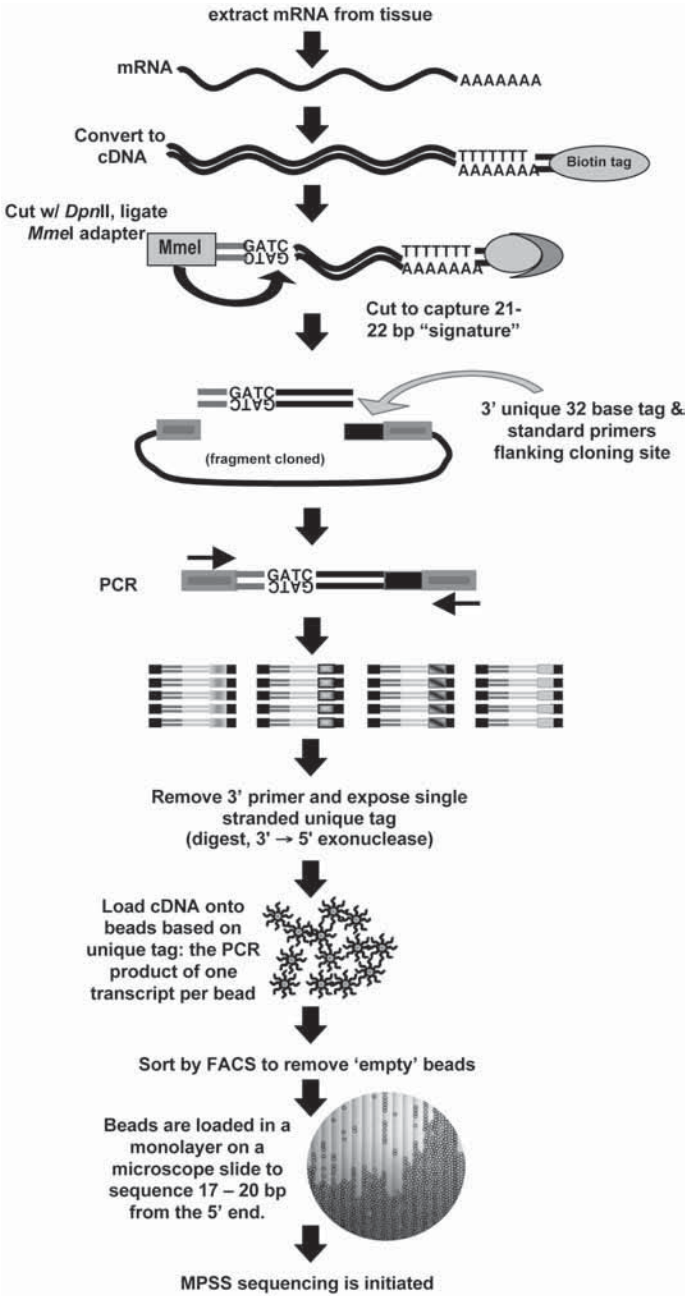

Fig. 1. (*continued from previous page*) stranded by treatment with an exonuclease, and the tagged cDNAs transferred by hybridization to microbeads. Each microbead is coated with a covalently linked, unique 32-base tags complementary to one of the cDNA combitags. This "loads" the beads with the tagged cDNAs, which are then enzymatically ligated. Each resulting microbead contains on its surface approx 100,000 identical cDNAs molecules derived from one mRNA transcript.

2. The cDNA is next digested with the restriction enzyme *Dpn*II (recognition sequence GATC) and the 3'-end fragments (cDNA fragments of *Dpn*II to poly-A sites) are affinity-purified using Streptavidin beads.
3. The 5' cDNA termini are ligated to an adapter containing a *Mme*I (Type IIS) restriction enzyme site. Cleavage with this enzyme generates a DNA fragment containing the adapter and a 20- or 21-bp portion of the cDNA, including the GATC *Dpn*II site.
4. The 3'-ends of these fragments are ligated to a second adapter, and these molecules are cloned directionally into a "signature cloning vector." This is a complex mixture of a single standard plasmid backbone that contains one copy out of 16.7 million different 32-bp "combitags" (*see* **Note 7**). This step generates a library of tag-signature clones, which is titered to determine its complexity.
5. From an aliquot of the library containing approx 1.3×10^6 signatures, the tag-signature molecules are amplified by PCR using a fluorescently labeled oligonucleotide. The combitag is made single-stranded by T4 polymerase treatment, and the product is then hybridized to 5-µm "microbeads." Each bead is coated with a distinct combitag complementary to one of the combitags adjacent to the signatures. The hybridization step associates the PCR product of each specific cDNA-derived signature with a single bead (approx100,000 identical copies of each signature per bead).
6. Only microbeads with attached cDNA fragments will possess the fluorescent label from the PCR, and these are physically separated from "unloaded" beads using a MoFlo high-speed cell sorter (Dako-Cytomation, Inc., Fort Collins, CO). The purified microbeads are loaded and immobilized in a monolayer array in a microfluidic flow cell as described in Brenner et al. *(4)*. This permits parallel sequencing of 20 nucleotides from each cDNA by MPSS.
7. The first step of sequencing is redigestion of cDNAs by *Dpn*II and ligation of an adapter molecule to this site; the adapter includes a *Bbv*I (Type IIS) restriction enzyme that cuts asymmetrically at positions 13 (5') and 9 (3') nucleotides away from the recognition site. The position of the *Bbv*I site results in a four-base single-stranded overhang immediately adjacent to the *Dpn*II site *(4)*, and these four nucleotides are the first to be sequenced (*see* **Note 8**).
8. A set of "encoded adapters" is ligated to the four-base overhang; the 5'-end of these adapters contains all 256 combinations of four nucleotides, the 3'-end contains 1 of 16 10-nucleotide single-stranded decoding sequences, and there is an internal *Bbv*I recognition site *(4)*. Four types of encoded adapters are ligated to each bead, each of which contains a unique decoder sequence that identifies one of the four nucleotides of the cDNA signature.
9. The presence on each bead of the 4 of 16 decoding sequences is determined by 16 hybridization steps, each using a different fluorescently labeled decoder probe. This process determines the identity of four nucleotides in the cDNA signature.
10. The sequencing cycle (**steps 8** and **9**) is repeated up to four times by digestion with *Bbv*I, removing the encoded adapter along with the four sequenced nucleotides, and exposing the adjacent four nucleotides for sequencing.

3.3.1. MPSS Image Acquisition and Base-Calling

1. A flow cell used for a single MPSS sequencing "run" contains approx 1.2 million beads; as mentioned in **step 6** in **Subheading 3.3.**, these beads are arranged in a two-dimensional array. The sequence information from **steps 7** to **10** is collected by recording and integrating the fluorescence signal on the beads with a charge-coupled device camera. The microscope objective magnification and the resolution of the camera define the number of beads visualized in a single image. Currently, each image covers 122nd of the useful surface of the flow cell and creates a 5 × 5 pixel area for each microbead. This results in approx 64,000 beads visualized in a single image, with 22 images or "tiles" for the entire flow cell. The images from each tile are analyzed and base-called independently.
2. The determination of the sequence of every bead occurs by successively hybridizing one of the 16 individual decoders (**steps 8** and **9** in **Subheading 3.3.**) and taking pictures corresponding to each tile by moving the modified microscope stage. This generates 16 × 22 (352) images for each four-nucleotide sequencing cycle. The sequencing protocol generates signatures 20 bases in length, necessitating 5 cycles × 352 images, for a total of 1760 images.
3. In addition to the fluorescent images, the sequencer takes a visible light (toplight) image of each of the 22 tiles at the beginning of the process to register the position of each bead in the different tiles. This is used to thread the bead's fluorescence across all the images collected across all the decoding steps for each cycle of four nucleotides of sequence information.
4. Once threading of the images is performed for each of the 16 images on a particular tile (for one sequencing cycle), there are three requirements for base-calling of an individual bead:
    a. A minimal fluorescence signal must be present.
    b. A minimal signal to local noise must be reached.
    c. The bead must have a minimal ratio of signal from one decoder to the next (3:1), to ensure that each bead has only one base at a particular nucleotide position.
5. Finally, all tiles from a single sequencing run are merged by summing the abundance count of identical sequences. This creates a final raw data set consisting of a list of distinct 17 or 20 base sequences with a raw abundance value representing the number of times that sequence was observed in the run.

3.4. Database Design and Implementation

We have designed a relational database for storage and handling of MPSS expression data and genomic sequence information. The schema for this database is shown in **Fig. 2**, and the genomic data are stored separately from the MPSS expression data. For parallel analysis of both the host and pathogen genome, the database should contain separate information from each genome.

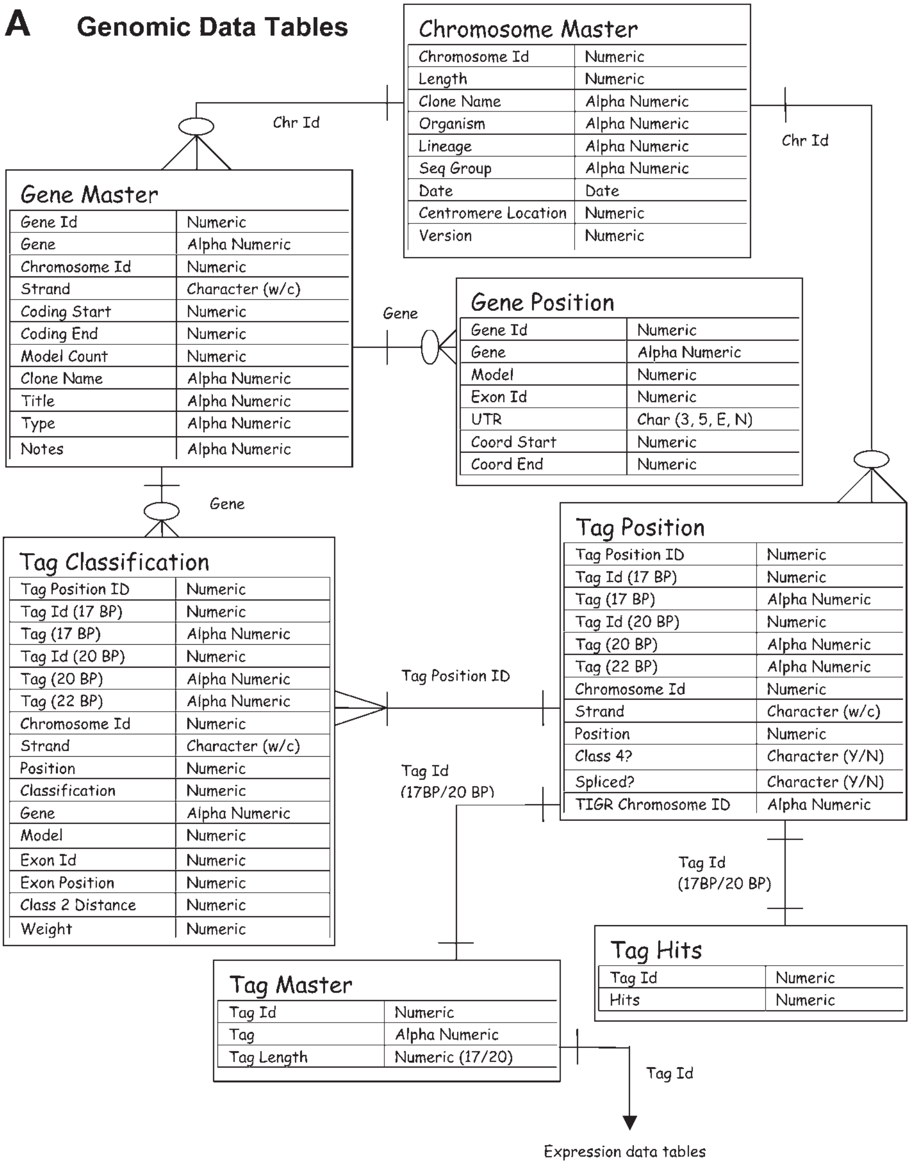

Fig. 2. Schema for a massively parallel signature sequencing (MPSS) database. The database is designed with two major sets of tables, one that contains the genomic annotation and genomic signature information and a second that contains the MPSS expression data. These genomic and expression data are linked through the "tag_master" table. The tables and fields are shown for each of the two major sets of tables. The lines connecting tables indicate one-to-one (simple lines) or one-to-many

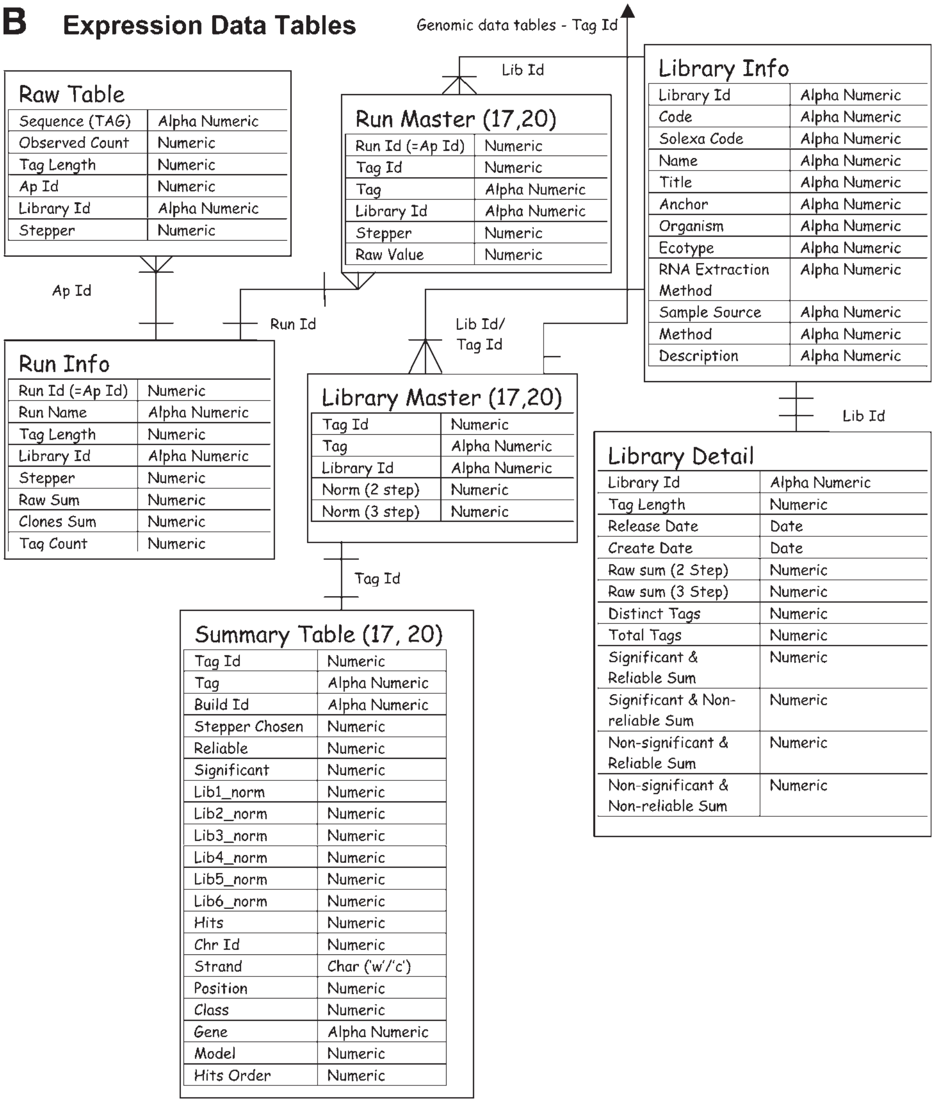

Fig. 2. (*continued from opposite page*)(branched lines) relationships. **(A)** The genomic data tables contain information extracted from available genome annotation files. These files are based on coordinates and include gene and exon information, as well as the extracted genomic signature sequences, coordinates and classification data. **(B)** The expression data tables contain information about the raw sequence data, the sequencing runs, libraries, as well as the normalized data. The most important information about each expressed signature, including the normalized abundance value in each library and the genomic information is stored in the "summary" table.

3.4.1. Genomic Data Tables

1. Genomic sequence and annotation data must be available; we typically use XML-formatted data from TIGR (http://www.tigr.org). Genomic tables indicated in **Fig. 2** include coordinate, strand and chromosome data for each gene, exon, or other genomic feature. Because the database build process is procedure-oriented, we typically build the primary tables (chromosome_master, gene_master, gene_position) using Oracle (Oracle Corporation, Redwood Shores, CA) and export these tables to MySQL for data analysis and our web interface.
2. The gene_master table is a master table for all genes. The gene_position table is a master table for all the exons in a gene. So for each gene, the gene_position table contains its exons, introns, and untranslated regions. An intermediate table named generic_gene_master is created using the information in both gene_master and gene_position to make sure that there is one table with all the relevant information from both gene_master and gene_position. This avoids "select" commands that require multiple tables and maximizes the database performance.
3. The "potential" or "genomic" signatures are extracted from the chromosome sequences. This uses a specialized script written in C++ that identifies each occurrence of "GATC" and copies the GATC plus 13 or 16 of 3' nucleotides into the "tag_position" table along with information on the chromosome, position, and strand of the sequence. Signatures must be extracted from both strands of the chromosome. Because our MPSS expression data includes signatures of both 17 and 20 bases, genomic signatures of these lengths are extracted and stored.
4. The next step is the classification of the genomic signatures. This step is the most important and time-consuming step of process of building the database. Each signature in the tag_position table is classified based on comparisons to the genome annotation, requiring the gene_master, gene_position and generic_gene_master tables. The classes are as follows: Class 1, in an exon, same strand as ORF; Class 2, the longer of either an annotated 3'-untranslated region or 500 bp after the stop codon, same strand as ORF; Class 3, antisense of an exon; Class 4, matching the genome but not class 1, 2, 3, 5 or 6 (in an intergenic region, for example); Class 5, entirely within and on the same strand as an intron; Class 6, entirely within an intron, but on the anti-sense strand; Class 7, signature includes an exon/intron boundary and is spliced. Signatures that are identified by MPSS but do not match to the genome are listed as "Class 0." All classified genomic signatures are stored in the tag_class table. Class 7 signatures (those that span annotated splice sites) must be identified using a separate signature extraction script because they are derived from spliced transcripts and not the raw genomic sequence. The process and classes of the signatures are described in more detail in Meyers et al. ***(6)***.
5. The tag_hits table contains distinct tags and the number of times it hits the genome (number of times it appears in the tag_position table). The tag_master is a list of all possible signature extracted from the genome, and after the expression data is added, the Class 0 signatures that do match the genome are added to this table.

3.4.2. Expression Data Tables

1. The expression data is received from Solexa in simple text files that contain, for each MPSS sequencing run, the signature sequenced by MPSS and a raw abundance level for each signature.
2. There are two primary sets of tables for the expression data. One set of tables includes the "run_master" table that contains a list of observed signatures and the abundance or expression level of those signatures found in each MPSS sequencing run. There are usually four runs per library, and each run is sequenced in a particular "stepper" *(4)*. However, the expression data represented in these tables requires additional processing to merge the runs and the steppers, and to produce a final normalized value in "transcripts per million" (TPM) for each signature in the library. The "library_master" table stores intermediate data in which the runs, but not the steppers, have been merged.
3. The normalized MPSS expression data for all of the libraries is stored in a single, large table; this "summary" table includes the results of two filtering steps and the merged sequencing runs. The steps in the construction of this table are described in more detail elsewhere *(6)*. In addition to the normalized expression data (in TPM), the "summary" table contains relational data that associate signatures with the genomic sequence and annotation. Much of these data are redundant with information stored in the other tables described in **Subheading 3.4.2.**, **item 2**, and **Fig. 2**, but genomic data for signatures duplicated in the genome is not stored in this table, nor are signatures found in the genome but not in the MPSS expression data. By creating a table that stores all of the required data, the disadvantages of data redundancy are outweighed by the improved functionality and enhanced performance of the database.

3.5. Graphic Interfaces for the Interpretation of MPSS Data

We developed web-based graphical interface and analysis tools specialized for MPSS data. The interface is written in PHP and requires the graphical library, GD. The interface accepts user inputs such as gene identifiers, query sequences, or chromosome position information to display the MPSS data matched to the genome. Although this is not an essential part of MPSS data analysis, the ability to visualize the data helps immeasurably in interpreting the results. The main entry page for our web site (http://mpss.udel.edu) provides an access point for MPSS data from different organisms.

3.6. MPSS Data Analysis

3.6.1. Statistical Analysis Methods

MPSS provides an absolute, rather than relative, count of the abundance of a specific transcript in a specific sample. This is a "digital" measurement that is conducive to relatively simple statistical tests, whereas the sample size of more than one million signatures per sample provides a high level of confi-

dence in statistical calculations. The observed abundance of a given signature in MPSS data demonstrates a binomial distribution, and statistical models based on such a distribution are applicable to MPSS data. For example, the *Z*-test model described by Man et al. ***(9)*** can be used to test if the level of a signature and the transcript from which it is derived is different in two samples. Under this model, if x_1 and x_2 represent the observed counts of a specific signature in samples 1 and 2, and n_1 and n_2 represent the total number of MPSS signatures sequenced from these samples, the proportions

$$p_1 = \frac{x_1}{n_1}$$

and

$$p_2 = \frac{x_2}{n_2}$$

each have a binomial distribution. Because n_1 and n_2 are large in MPSS (typically $>10^6$), the difference $(p_1 - p_2)$ follows an approximate normal distribution, defined as:

$$\boldsymbol{N}\left(\left(\boldsymbol{p}_1 - \boldsymbol{p}_2\right), \sqrt{\boldsymbol{pq}\left(\frac{1}{\boldsymbol{n}_1} + \frac{1}{\boldsymbol{n}_2}\right)}\right)$$

where the unknown parameters p and q can be estimated as

$$\hat{p} = \frac{x_1 + x_2}{n_1 + n_2}$$

and,

$$\hat{q} = 1 - \hat{p}$$

respectively. In the following normally distributed statistical test, λ can be used with standard statistical tables to determine the *Z* score and the *p* value, providing an estimate of confidence in the difference between the signature abundance in the two samples:

$$\lambda = \frac{p_1 - p_2}{\sqrt{\hat{p}\hat{q}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

with $p < 0.001$, a twofold difference in expression can be detected for genes expressed at only 30 to 40 TPM, or smaller changes may be detected for genes

expressed at a higher levels. Although the *Z*-test is suitable for most analyses, alternative statistical models have been described for use with MPSS data that are more stringent ***(10)***.

The *Z*-test allows MPSS users to quickly identify differentially expressed genes between two samples. However, like many other statistical tests for pairwise comparison, the *Z*-test has limited analytical power when applied to multiple samples. Because of the digital nature of MPSS data, direct comparison of large numbers of samples is feasible. We have found that most commercial software packages that were originally designed for analyzing microarray data are equally useful in analyzing MPSS data sets.

3.6.2. The Use of Commercial Gene Expression Packages for MPSS Analysis

Numerous software packages are commercially available for the analysis of gene expression data. These tools can be applied to MPSS data by converting the MPSS data into the correct input file format; typically this requires summing the abundance of the signatures for each gene and entering the data based on gene identifier numbers. These software packages include Spotfire, Resolver, Partek Pro, and GeneSpring. These packages facilitate analyses such as principal components analysis, hierarchical clustering, self-organizing maps, data filtering, pathway views, etc.

3.6.3. Gene Inventories and Analysis of Tissue Specificity

A basic application of expression analysis using MPSS is to generate “inventories” of expressed genes. This allows the user to catalog nearly every gene found in a specific tissue or treatment, and sort these based on absolute abundance level. As the database increases with the addition of more libraries, repeated identification of the same transcript across tissues will validate the expression of that gene. Because sequence-based technologies for measuring gene expression, including estimated sequence tags, SAGE, and MPSS, require no previous knowledge of expressed transcripts, it is possible to discover novel transcripts that may play an important role in the biology of the sample that is being studied. These data can be used to annotate genomic sequence.

Another application of the data is the identification of regulatory sequences with specific expression characteristics. We have analyzed diverse *Arabidopsis* tissues to identify genes that show evidence of tissue specificity or low, moderate, or high levels of expression ***(11)***. The promoters of genes identified through such an approach may have useful experimental characteristics, and the genes may be good markers for the specific trait of interest.

3.7. Separating Expressed Signatures Derived From the Host or Pathogen

Sequence-based technologies such as MPSS are sensitive to sequence differences. With the genomic data for both a host and pathogen, it is possible to separate signatures derived from either organism. As described previously, the potential or genomic signatures can be extracted from both host and pathogen, and by comparing these two sets of sequences, it is possible to determine signatures that uniquely map to each genome or will map to both genomes; only signatures in the former category will be useful, but given that most host–pathogen interactions are across kingdoms, relatively few signatures are expected to be perfectly conserved across the two genomes. Most signatures matching both genomes will do so purely by chance and rarely because they are conserved. MPSS data derived from infected material with a eukaryotic host and pathogen will simultaneously measure host and pathogen gene expression.

4. Notes

1. Use polypropylene tubes (such as standard microfuge tubes) or glass, because some types of plastics will dissolve after treatment with phenol or chloroform.
2. The TRIZOL reagent is hazardous; be sure to wear gloves, eye protection, and a laboratory coat.
3. Homogenization can be performed using a mortar and pestle or, for larger volumes, a machine such as a Polytron can be used. Using a mortar and pestle, it may be easier to grind the frozen material without the TRIZOL, transfer the powdered tissue to a tube, then immediately add the TRIZOL; in a mortar prechilled with liquid nitrogen, the TRIZOL will solidify. For larger volumes, 10 mL of TRIZOL can be used per 1 g of tissue, with the later steps using 50-mL conical tubes centrifuged at 12,000*g*.
4. A chilled centrifuge at approx 4°C is preferable but not essential in each of the centrifugation steps.
5. This step is listed as optional in some protocols; because many plant tissues contain high levels of polysaccharides or extracellular material, we routinely perform this step in our extractions to avoid problems in later stages.
6. Dispose of TRIZOL and chloroform appropriately because they are hazardous chemicals.
7. As described in Brenner et al. *(5)*, the 32 nucleotide combitags are produced by eight rounds of combinatorial synthesis using combinations of the four-nucleotide words CATT, TCAT, TACA, TTTC, CTAA, ACTA, ATCT, and AAAC. These tags are isothermal melting temperatures and the mixture is complex enough such that each cDNA is ligated to a unique combitag.
8. Samples are typically sequenced in two frames ("steppers") by the use of initiating adapters in which the *Bbv*I site is offset by one or two bases (also described in Meyers et al. *[6]*).

Acknowledgment

The methods described here were developed with support from the National Science Foundation Plant Genome Research Program.

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Use of Microarray Analysis to Dissect the Plant Defense Response

Jane Glazebrook

Summary

Microarray analysis is a technology that allows simultaneous measurement of the messenger RNA levels of thousands of genes. There are several different technology platforms in use, including oligo arrays synthesized directly on the underlying substrate, and spotted arrays produced by applying oligonucleotides or other nucleic acids to glass slides. The advantages of various platforms are discussed. Analysis of the large data sets produced from microarray experiments requires the application of statistical methods to define significant differences in gene expression, and computerized algorithms for pattern recognition. Early applications of microarray analysis to studies of disease resistance have led to recognition of the large numbers of genes that respond to infection, insights into the nature of gene-for-gene resistance, efforts to model the topology of the signaling network controlling inducible defense responses, and identification of promoter elements associated with particular expression patterns.

Key Words: Microarray; expression profile; resistance gene; statistical analysis; signaling.

1. Introduction

Plants respond to pathogen attack by activation of a large number of inducible defense mechanisms. This response includes increased transcription of many genes. The fact that rapid activation of gene expression is correlated with resistance suggests that the identification of genes that undergo expression changes in response to pathogen attack and the elucidation of the signal transduction mechanisms that control their expression are essential for understanding how plants defend themselves from pathogens. Consequently, gene-expression studies have long been a major component of research aimed at understanding the molecular basis of disease resistance.

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

The development of genome-scale microarrays has provided a powerful new tool for gene expression studies. Microarrays consist of dense arrays of nucleic acid probes attached to a substrate such as a glass slide. They can represent tens of thousands of genes, which is the entire genome for many organisms. The expression levels of essentially every gene in a genome can be monitored simultaneously by hybridizing fluorescently labeled RNA preparations to the arrays. As discussed in this chapter, microarrays have been used for discovery of pathogen-induced genes, for studies of the nature of gene-for-gene resistance, in efforts to build genetic models of the signal transduction circuitry controlling defense gene expression, and for identification of promoter elements that may mediate coordinated expression of defense genes.

2. Materials

2.1. Choice of Platform

Several different microarray platforms are in widespread use. Some arrays cannot be easily manufactured on site and therefore they must be purchased from commercial entities. Others are produced by spotting nucleic acids on glass slides, using equipment that is widely available. Different platforms have different advantages and disadvantages that affect their suitability for specific microarray experiments.

Affymetrix produces GeneChip® oligonucleotide arrays (www.Affymetrix.com). These arrays consist of oligonucleotides synthesized directly on the arrays. Each oligonucleotide occupies a very small space, so one array can include more than 500,000 different oligonucleotides. This feature allows each gene to be represented by multiple oligonucleotides. Typically, one gene is represented by 16 to 20 oligonucleotides that correspond perfectly to the target gene, and a corresponding set of oligonucleotides each containing a single base mismatch. The expression level for each gene on the array is calculated by combining the signals from the perfect match oligonucleotides, correcting for nonspecific hybridization using the mismatch oligonucleotides, correcting for local background, and normalizing over the entire array. Thus, the gene expression level is provided as the expression level relative to the rest of the genes on the array. For many species, GeneChip arrays have the capacity to represent the entire genome; therefore, they are very useful for experiments aimed at discovery of all genes that show certain expression patterns. Of course, such discovery is limited by the sensitivity of the arrays; genes expressed at very low levels or in only a few of the cells composing a sample cannot be detected. GeneChip arrays have excellent technical reproducibility because of their consistency in manufacturing and the statistical power resulting from having multiple measurements for each gene. However, GeneChip arrays are only available for certain organisms. The design of the arrays cannot be altered by

individual investigators because the initial costs of production of a new array are very great. GeneChip experiments also are expensive relative to some other types of arrays; therefore, the costs of using them for large numbers of samples tend to become prohibitive.

Arrays produced by Nimblegen are similar to GeneChip arrays in that the oligonucleotides are synthesized directly on the array at extremely high density and that each gene is represented by multiple oligonucleotides (www.nimblegen.com). The major advantage of this platform is that a different manufacturing method allows the design of the arrays to be readily changed so that the use of custom-designed Nimblegen arrays by individual investigators may not be prohibitively expensive. To use these arrays, customers supply Nimblegen with RNA samples; the company conducts array production and hybridization and returns the data. Using Nimblegen arrays is slightly more expensive than using GeneChips and, therefore, cost considerations tend to restrict use of this platform to experiments requiring relatively small numbers of samples.

Arrays also may be produced by spotting nucleic acids onto glass slides. The equipment required for doing this is widely available at research universities. The density of such arrays is not as high as those of GeneChips or Nimblegen arrays, so each gene is usually represented by only one or two spots. The spots may consist of long (60–70 mer) oligonucleotides, polymerase chain reaction (PCR) products, or complementary DNA (cDNA). Long oligonucleotides have the considerable advantage that they avoid the labor required for production of thousands of PCR products or cDNA clones. Also, it is easier to distinguish members of gene families using long oligonucleotides, as they require shorter stretches of gene-specific sequence. However, the larger target sizes of PCR products and cDNA clones can result in increased sensitivity relative to long oligonucleotides.

Spotted arrays generally show substantial array-to-array variation because of low uniformity in spotting. Consequently, these arrays are normally used with "two-color" methods. Two samples are labeled with different dyes and hybridized to the same array. The signal is expressed as the ratio between the two signals. If the investigator is interested in comparing multiple samples to each other, the experiment should be designed accordingly. One strategy is to use a single control sample labeled with one dye together with each of the experimental samples labeled with another dye. This control sample could be an RNA sample or a genomic DNA sample. In this case, the desired ratios can be derived computationally. Consider two arrays, one probed with sample A and reference R and the other probed with sample B and reference, R. The ratio of A to B can be derived as $(A/R) \times (R/B)$. This method increases the error in the measurement of A/B relative to a measurement obtained by applying A and B to the same array. Nevertheless, it is useful for experiments that require com-

parisons among many samples in many combinations, as it reduces the numbers of arrays required.

There are several advantages to using spotted arrays. They offer great flexibility in design, allowing custom arrays to be produced as needed for specific applications. They are suitable for experiments requiring arrays representing a few hundred genes as well as for arrays representing tens of thousands of genes. They are relatively inexpensive to produce and use, and the required equipment for doing this is widely available. The major disadvantage is that technical reproducibility is generally poorer than that of GeneChips, because of variations in spotting and reduced statistical power resulting from the use of only one or two spots to represent each gene. Data quality can be improved by using multiple technical replicates for each sample, which may be less expensive than using a single GeneChip array. Also, for small arrays it is feasible to spot the entire array multiple times on each slide, thereby obtaining multiple measurements for the expression level of each gene and increasing statistical power.

3. Methods

3.1. Statistical Considerations

Statistical analysis is important for many aspects of microarray data analysis. The primary data from a microarray experiment is an image showing variations in fluorescence intensity over the surface of the array. Conversion of this image into an expression measurement for each gene represented by the array is usually accomplished using a commercial software package that integrates the intensity of each pixel over the area occupied by each array element, corrects for local background, and in the case of arrays with multiple elements for each gene, combines the data from each element into a single measurement. The data are then normalized to compensate for variations in labeling efficiency between samples. These operations do not generally require decision making by the investigator, so they are not discussed further here.

A common question addressed by microarray experiments is, "Which genes are expressed at different levels in pathogen-infected tissue than in uninfected tissue?" This apparently simple question is actually quite difficult to answer with confidence. The difficulty arises from random variations that affect both the actual expression levels of genes and the measurements of expression levels, combined with the large number of genes tested. Consider a simple experiment with three biological replicates of mock-infected and infected plants. Various statistical tests can be applied to select genes that are expressed at different levels in infected than in mock-infected plants at 95% confidence (*see* **Note 1**). However, if the array represents 10,000 genes, then 5% of these, or 500 genes, can be expected to pass the test even though they are not truly differentially expressed. Imposing more stringent statistical criteria reduces the

number of such false-positives, but necessarily also increases the number of false-negatives. Consequently, investigators must keep the limitations of the analyses in mind, and tailor statistical methods according to the goals of the work. For some purposes, it is better to reduce false-positives (for example, when the goal of the experiment is to identify a few reliable expression changes correlated with infection), whereas for others it is better to reduce false-negatives (for example, when the goal is to obtain an expression profile (*see* **Note 2**) to compare with profiles obtained after infection by other pathogens).

Expression profiles also can be viewed as detailed descriptions of cell states. Similarities among expression profiles suggest similar host responses to different pathogens, genes that act at similar points in genetic regulatory networks, and groups of genes with similar biological functions. Identification of similarities among expression profiles is a pattern recognition problem in a high-dimensional space (*see* **Note 3**). Computational methods are required to identify the patterns and to display them in a form that is readily perceived by humans. Hierarchical clustering is one such method that arranges both genes and experiments according to similarities in expression patterns ***(1)***. The software is freely available (http://rana.lbl.gov/EisenSoftware.htm). Similarity relationships among genes and experiments are represented as tree diagrams that are familiar to biologists as they are similar to the tree diagrams used to represent phylogenetic relationships. Gene expression levels are represented using a simple two-color scale (red/green is a common choice) that allows investigators to perceive patterns in the data easily. The major advantages of hierarchical clustering are its simplicity and its easily visualized output. However, it suffers from some limitations. It is fundamentally a one-dimensional method. It only shows which profiles are most similar to each other overall; it cannot reveal that two profiles are both similar to a third, but in different ways. It is also strongly affected by the sequence of the pair-wise clustering events that lead to the final tree. As a result, small changes in the compositions of the profiles can have dramatic effects on the structures of the trees. Some of these difficulties can be overcome by using other clustering methods such as self-organizing maps ***(2)***, K-means clustering ***(3)***, or principal component analysis ***(4,5)***. A method based on nonlinear dimensionality reduction ***(6)***, called local context finder ***(7)***, may prove useful for multidimensional pattern recognition in complex data sets.

3.2. Examples of Microarray Analyses of Plant Defense Responses

Relatively few microarray studies of plant–pathogen interactions have been published, but even these early studies demonstrate that microarray experiments can be used to address questions that are otherwise difficult to approach. A very basic question is, “Which plant genes undergo expression changes in response

to pathogen attack?" The answer seems to be that an enormous number of genes undergo expression changes. From various experiments conducted using an Affymetrix chip representing 8000 *Arabidopsis* genes, 500 to 2000 genes showed expression changes, which is a large fraction of the genome. It is quite possible that some of these genes are not responding to the pathogen attack directly but rather are responding to insults to homeostasis that occur as a consequence of pathogen activity. However, extensive gene expression changes are observed at very early times after infection, when it seems unlikely that the pathogen would have had much effect on host metabolism ***(8,9)***.

A common motivation for searching for genes induced in response to infection is the idea that genes that show such induction are likely to be involved in resistance. Of course, this is not necessarily true. Further work is needed to determine whether or not a pathogen-induced gene contributes to resistance. Fortunately, the development of powerful reverse genetics methods, including large transfer DNA insertion collections ***(10,11)*** and RNAi technology ***(12)***, has made it feasible to conduct functional assays on quite large numbers of candidate genes. Finding that knocking out a candidate gene by mutation or RNAi results in reduced resistance is very good evidence for a role for that gene in resistance.

Expression profiling was used to investigate the molecular basis of induced systemic resistance (ISR), a jasmonate and ethylene-dependent, salicylate-independent resistance induced by root-colonizing rhizobacteria ***(13)***. No significant changes in gene expression were associated with development of ISR in the absence of pathogen challenge ***(13)***. After challenge, ISR plants responded more rapidly to pathogen challenge than naïve plants, suggesting that ISR is a consequence of potentiation of defense responses ***(13)***.

Studies of expression profiles have led to new insights about gene-for-gene resistance. Tao et al. ***(9)*** found that the shapes of the profiles from plants undergoing gene-for-gene resistance in response to *Psm* ES4326/*avrRpt2* were very similar to those of plants responding to the virulent strain *Psm* ES4326. However, in the case of the avirulent strain, the amplitudes of the profiles were much greater. The amplitude of the profile from plants responding to *Psm* ES4326 at 30 h after infection was similar to those of plants responding to avirulent strains at 6 h after infection. This indicates that many defense responses are common to basal resistance and gene-for-gene resistance, with the major difference between the two lying in the kinetics and/or intensity of defense activation, consistent with the suggestion made by Lamb et al. years ago ***(14)***.

R genes vary considerably with respect to genetic requirements for effective resistance. For example, *RPP4* requires PAD4, SA accumulation and SGT1b ***(15)***; *RPP7* requires SGT1b but is independent of PAD4 and SA ***(16)***, whereas

RPP8 is independent of SGT1b, PAD4, and SA ***(17)***. One interpretation of these results is that these three *R* genes trigger activation of different defense mechanisms that are under the control of different downstream signaling genes. However, this does not appear to be the case, because the expression profiles of resistance reactions triggered by *RPP4*, *RPP7*, or *RPP8* are all very similar ***(18)***. Rather than triggering different defense responses, the three *R* genes seem to require different signaling elements to achieve very similar results, suggesting a convergence point in the signaling pathways triggered by each *R* gene product ***(18)***.

In principle, expression profiles should be useful in efforts to model genetic regulatory networks controlling gene expression. Wild-type and various regulatory mutant plants were subjected to expression profiling after infection with *Psm*ES4326. From the data, it was possible to group mutations affecting SA and JA/ET signaling based on profile similarity ***(19)***. The roles of four genes, *PAD1*, *PAD2*, *EDS3*, and *EDS8*, in signaling were predicted based on the expression profiles of the mutants. *PAD1* and *EDS8* were correctly predicted to affect JA signaling, and *EDS3* was correctly predicted to affect SA signaling. A prediction that *PAD2* affects SA signaling could not be verified. These results are encouraging, but more sophisticated methods for pattern recognition are needed to derive high-resolution information about signaling network structure from expression profiling data. Multidimensional pattern recognition methods such as local context finder may be helpful in this regard ***(7)***.

Genes that are coregulated are presumably controlled by similar sets of transcription factors that bind to conserved sites in promoters. Several studies have defined promoter elements that are conserved among groups of co-regulated genes. Binding sites for WRKY transcription factors are present at substantially higher frequencies in SA-regulated genes than in the rest of the genome ***(20)***. They also are enriched in promoters of transcription factor genes that are induced in response to infection ***(21)***. Promoters of genes induced in response to *RPP4*, *RPP7*, and *RPP8* activation were enriched for motifs associated with binding of WRKY-, TGA-, and ERF-type transcription factors ***(18)***. These studies provide useful clues about transcriptional activation of defense-related genes that can serve as a guide for further research.

3.3. Concluding Remarks

Microarray analysis is a relatively new technology. Methods for array production and hybridization are still improving. The analysis of microarray data requires statistical analyses that are unfamiliar to many biologists. These methods must be tailored to fit the goals of particular experiments. The results from recent applications of microarray analysis to studies of plant disease resistance illustrate the tremendous potential of this technology. The use of expression

profiling as a phenotyping tool is a particularly powerful application. Improvements in methods for pattern recognition in expression profiling data can be expected to facilitate systems-level investigations into plant defense responses.

4. Notes

1. Several different statistical tests are commonly used to identify differentially expressed genes using microarray data. The small number of replicate data sets that are usually available presents challenges for tests such as *t*-tests, which are commonly used. The permutation testing method, called significance analysis of microarrays (SAM), is probably better for handling small numbers of replicates ***(22)***. SAM has the additional advantage of providing an estimate of the false discovery rate, which is helpful in deciding where to set the cut-off between genes judged to be differentially expressed and genes judged not to be. SAM software is freely available (http://www-stat.stanford.edu/~tibs/SAM/). Development of statistical methods for detecting differentially expressed genes in microarray data is an area of active research. It is quite possible that there are methods better than SAM that the author is unfamiliar with.
2. A single microarray experiment yields expression level values for many genes from a particular sample. Collectively, these data constitute a snapshot of the expression pattern of the genome for that sample. This snapshot is called the expression profile of that sample.
3. Pattern-recognition programs represent expression profiles in n-dimensional space, where n is the number of genes represented in the profiles. Each profile is defined as a vector in the space, with the end point of the vector determined by the expression values of each gene in the profile. This is easy to imagine when each profile consists of data for only three genes. You can plot the vectors using three axes. When asked which vectors are most similar, you can easily answer based on which vectors are closest to each other in the three-dimensional space. The mathematics behind the problem do not change when the space has more dimensions, but humans cannot easily visualize the arrangements of the vectors.

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13

Use of Robust-Long Serial Analysis of Gene Expression to Identify Novel Fungal and Plant Genes Involved in Host–Pathogen Interactions

Malali Gowda, Venu Reddyvarichannarayappa, Yulin Jia, Eric Stahlberg, Vishal Pampanwar, Carol Soderlund, and Guo-Liang Wang

Summary

Identification of important transcripts from fungal pathogens and host plants is indispensable for full understanding the molecular events occurring during fungal–plant interactions. Recently, we developed an improved LongSAGE method called robust-long serial analysis of gene expression (RL-SAGE) for deep transcriptome analysis of fungal and plant genomes. Using this method, we made 10 RL-SAGE libraries from two plant species (*Oryza sativa* and *Zea maize*) and one fungal pathogen (*Magnaporthe grisea*). Many of the transcripts identified from these libraries were novel in comparison with their corresponding EST collections. Bioinformatic tools and databases for analyzing the RL-SAGE data were developed. Our results demonstrate that RL-SAGE is an effective approach for large-scale identification of expressed genes in fungal and plant genomes.

Key Words: Robust-LongSAGE (RL-SAGE); *Oryza sativa*; *Magnaporthe grisea*; disease resistance; *Zea maize*; *Rhizoctonia solani*.

1. Introduction

Many fungal genes play an important role in their pathogenic effects on host plants. Similarly, many host genes participate in the defense response to fungal infection. Identification and characterization of these fungal and host genes will lead to a better understanding of the molecular events during fungal–plant interactions. In the recent years, several large-scale genomics approaches have been developed for transcriptome analysis of plant and fungal genomes. These methods include expressed sequenced tag (EST) sequencing (*1,2*), micorarray (*3,4*), serial analysis of gene expression (SAGE [*5,6*]), and massively parallel signature sequencing (MPSS [*7,8*]). Compared with SAGE and MPSS meth-

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

ods, EST and microarray procedures are relatively simple techniques for expression analysis. EST sequencing was the first method for gene discovery and expression analysis, and it has been recently used in studies on fungal–plant interactions (*1,2,9*). However, high levels of gene redundancy in the EST sequences and the inability to detect low-abundance transcripts has limited that method for deep transcriptome analysis (*2*). Microarrays are an efficient tool for profiling and they have been used in many plant–microbe interaction studies (*4*). One of the disadvantages of microarrays is that a large set of EST or genomic sequences must be available for the microarray design. In addition, RNA variants, such as alternative splicing transcripts, cannot be detected using microarrays (*10*). Recently, MPSS has been used to characterize the *Arabidopsis* genome, and many other genes uncharacterized by ESTs sequencing were identified (*8*). However, MPSS libraries can only be constructed by Solexa (http://www.solexa.com), and the MPSS technique is exclusively licensed to a company for several important crop plants.

Compared with the aforementioned methods, SAGE is a unique method that is not only ideal for large-scale expression profiling but is also easy to use in any molecular laboratory (*5*). SAGE is based on two basic principles: isolation of a short sequence tag (14 or 21 bp) from the 3' region of a transcript and the concatenation of multiple tags in a serial fashion for sequencing (*5,11*). It is a high-throughput method for evaluating the different levels of transcripts without previous sequence information. Of importance, the transcript variations from alternative initiation and termination, alternative splicing, trans-splicing, and antisense transcription can be revealed by using SAGE (*10,12*).

In the last decade, SAGE has been applied mainly in mammalian systems (*13,14*). There have only been several plant SAGE libraries reported because of some difficulties in the library construction. Low cloning efficiency and the small inserts of SAGE clones are two major problems in SAGE library construction. Recently, we developed an efficient and rapid method called robust-long serial analysis of gene expression (RL-SAGE) that solved these two problems (*6*). Using our RL-SAGE protocol, 10 libraries of rice blast fungus *Maganporthe grisea*, rice, and maize plants were made. Here, we report the major steps for RNA preparation from fungal and plant tissues, RL-SAGE library construction, di-tag and individual tag extraction, annotation and matching of RL-SAGE tags, and digital display of RL-SAGE tags in the *Magnaporthe grisea Oryza sativa* (MGOS) database. The methods optimized for fungal and plant RL-SAGE library construction and analysis in our laboratory should be easily applied to other organisms (*see* **Note 1**).

2. Materials

2.1. Fungus Infection Assay

2.1.1. M. grisea *Infection*

1. An avirulent isolate C9240 (from H. Leung, International Rice Research Institute, Philippines).
2. A virulent strain Che86061 (from G. Lu, Fujian Province, China).
3. Nipponbare seeds.
4. Conviron growth chamber.
5. Oatmeal agar (50 g of oatmeal +15 g of agar).
6. Conidiospores.
7. 0.01% Tween-20.
8. Plastic container.
9. Parafilm.
10. Distilled water.
11. Microscope.
12. Microscopic slides and cover slips.
13. Hemocytometer.

2.1.2. Rice Sheath Blight Infection Assay

1. Detached leaves.
2. Filter papers.
3. Petri plates.
4. *Rhizoctonia solani* strain RR0102.
5. Jasmine 85 seeds.
6. Water agar.

2.1.3. M. grisea *Mycelia Liquid Culture*

1. Strain 70–25 (from Ralph Dean, North Carolina State University).
2. Liquid medium: 0.2% (w/v) yeast extract and 1% (w/v) sucrose for 3 d (28°C at 200 rpm.

2.1.4. Isolation of Total RNA and Messenger RNA From M. grisea *Mycelia and Infected Rice Leaf Tissue*

1. Liquid nitrogen.
2. Trizol solution (Invitrogen, Carlsbad, CA).
3. Chloroform.
4. Iso-propanol.
5. 95% and 75% ethanol.
6. Diethyl pyrocarbonate (DEPC)-treated H_2O.
7. mRNA isolation kit (Qiagen Inc., Valencia, CA).

2.1.5. RL-SAGE Library Construction

1. I-SAGE/I-LongSAGE kit with magnetic stand (Invitrogen, Carlsbad, CA).
2. Siliconized (non-sticky) 1.5-mL tubes (Ambion, Inc, Austin, TX).
3. Streptavidin beads (Dynal Biotech Inc., Lake Success, NY).
4. *Nla*III and *Mme*I (New England Biolabs, Inc., Beverly, MA).
5. PAGE-purifiedd oligos (Integrated DNA Technologies Inc, Coralville, IA ***[11]***).
   Linker 1A:
   5'-TTTGGATTTGCTGGTGCAGTACAACTAGGCTTAATATCC GACATG-3'
   Linker 1B:
   5'-TCGGATATTAAGCCTAGTTGTACTGCACCAGCAAATCC-C7 amino-modified-3'
   Linker 2A:
   5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGTCCGACATG-3'
   Linker 2B:
   5'-TCGGACGTACATCGTTAGAAGCTTGAATTCGAGCAG-C7 amino-modified-3'
6. PCR primers ***(11)***:
   Primer 1: 5'-biotin GTGCTCGTGGGATTTGCTGGTGCAGTACA-3'
   Primer 2: 5'-biotin GAGCTCGTGCTGCTCGAATTCAAGCTTCT-3'
7. Magnetic stand (Invitrogen, Carlsbad, CA).
8. Dynal oligo(dT) magnetic beads (5 mg/mL in phosphate-buffered saline containing 0.02% sodium azide; *see* I-SAGE kit instructions).
9. Wash buffer A: 10 m*M* Tris-HCl, pH 7.5 0.15 *M* LiCl, 1 m*M* ethylene diamine tetraacetic acid (EDTA), 0.1% lithium dodecyl sulfate, 10 μg/mL glycogen (*see* I-SAGE kit instructions).
10. Wash buffer B: 10 m*M* Tris-HCl, pH 7.5, 150 m*M* LiCl, 1 m*M* EDTA, 10 μg/mL glycogen (*see* I-SAGE kit instructions).
11. Wash buffer C: 5 m*M* Tris-HCl, pH 7.5, 0.5 m*M* EDTA, 1 *M* NaCl, 1% sodium dodecyl sulfate, 10 μg/mL mussel glycogen (*see* I-SAGE kit instructions).
12. Wash buffer D: 5 m*M* Tris-HCl, pH 7.5, 0.5 m*M* EDTA, 1 *M* NaCl, and 200 μg/mL bovine serum albumin (*see* I-SAGE kit instructions).
13. Lysis/binding buffer: 100 m*M* Tris-HCl, pH 7.5, 500 m*M* LiCl, 10 m*M* EDTA, 1% lithium dodecyl sulfate, and 5 m*M* dithiothreitol (*see* I-SAGE kit instructions).
14. SOC medium: 0.5% yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $MgSO_4$, 20 m*M* glucose.
15. Zeocin antibiotic (100 mg/mL in water; *see* I-SAGE kit instructions).
16. TE buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0.
17. pZero-1 (1 μg/μL; *see* I-SAGE kit instructions).
18. Platinum *Taq* DNA polymerase (Invitrogen).
19. Phenol:chloroform:isoamyl alcohol (25:24:1,v/v).
20. Vertical gel electrophoresis apparatus for large format gels (15 × 17 cm).
21. 40% (w/v) acrylamide:bisacrylamide solution (29:1).

22. 10% (w/v) ammonium persulfate.
23. TEMED.
24. 5X TBE running buffer: 89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA ***(15)***.
25. 5X TBE sample buffer: 18 m*M* Tris base, 18 m*M* boric acid, 0.4 m*M* EDTA, 3% Ficoll Type 400, 0.02% bromophenol blue, 0.02% xylene cyanol ***(15)***.
26. 0.5-mm Spacer and comb.
27. One-shot TOP10 electrocompetent cells (Invitrogen).
28. Low-salt luria broth agar plates with Zeocin composition: 1% Tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5.
29. 7.5 *M* Ammonium acetate.
30. Freeze medium: 2.5% w/v luria broth, 13 m*M* KH_2PO_4, 36 m*M* K_2HPO_4, 1.7 m*M* sodium citrate, 6.8 m*M* $(NH_4)_2SO_4$, and 4.4% v/v glycerol.

3. Methods

3.1. Leaf Tissue From M. grisea-Infected Rice Plants (Nipponbare)

1. Grow Nipponbare seedling for 21 d in a Conviron growth chamber at 26°C during the day, 80% relative humidity, 12 h light at 200 μmol photons m^{-2} s^{-1} and 20°C at night, 60% relative humidity.
2. Collect ascospores from *M. grisea* isolate Che86061 (virulent) or C9240–2 (avirulent) mycelium, which has grown on oatmeal agar plates for 2 wk.
3. Inoculate the experimental plants with 2×10^5 spores/mL in 0.01% Tween-20 solution.
4. Inoculate the control plants with only the 0.01% Tween-20 solution.
5. Keep the inoculated plants in a sealed plastic container in the dark for 24 h with 100% humidity and then move the plants to normal growth chamber conditions for 5 to 6 d for disease development.
6. Harvest-infected leaves at 24 and 96 h after inoculation for RNA isolation.

3.2. Leaf Tissue for R. solani Infection (Jasmine 85)

1. Grow Jasmine 85 plants to V11 stage ***(16,17)*** in a greenhouse.
2. Detach approx 14 cm of the second youngest leaves of the rice plants and immediately place in a plastic container (24 × 24 × 1.8 cm) with wet filter papers (23 × 23 cm).
3. Use a 1-mL Eppendorf tip to excise a 0.8-cm-diameter potato dextrose agar plug containing mycelium of *R. solani* isolate PR0102 and remove the plug with a sterile toothpick.
4. Place the 0.8-cm-diameter agar plug on the abaxial leaf surface of the collected leaves and seal the container with parafilm to maintain high humidity. For control leaves, use a 0.8-cm-diameter potato dextrose agar plug without any pathogens for inoculation.
5. Keep the container under cool white fluorescence light at 21 to 24°C. Harvest leaf tissue near the infection area for RNA isolation after 16 h of inoculation.

3.3. M. grisea *Mycelia Tissue From In Vitro Culture*

1. Inoculate fungus (70–25 mycelium) in a liquid medium (0.2% [w/v] yeast extract and 1% [w/v] sucrose).
2. Shake for 3 d at 28°C at 200 rpm.
3. Centrifuge the medium to collect the mycelium tissue, and freeze it in liquid nitrogen for RNA isolation.

3.4. *Isolation of Total RNA and Purification of mRNA*

1. Grind approx 2 g of leaf or mycelium tissue into a fine powder using liquid nitrogen and immediately transfer into 15 mL of Trizol solution.
2. Mix well and incubate at room temperature for 10 min.
3. Add 4 mL of chloroform, incubate at room temperature for 5 min, and then centrifuge for 20 min (9000*g*) at 4°C.
4. Transfer supernatant into another 25-mL tube containing 10 mL of ice-cold isopropanol, mix well, and then incubate on ice for 10 min.
5. Centrifuge for 15 min (9000*g*) at 4°C.
6. Wash the RNA pellet with 15 mL of 75% ethanol, centrifuge for 10 min (9000*g*), and then discard the alcohol.
7. Dry the RNA pellet at room temperature for 10 to 15 min.
8. Dissolve the RNA pellet in 700 µL of DEPC-treated H_2O at 65°C for 10 min.
9. Quantify the amount of total RNa using a spectrophotometer by taking OD at 260/280 nm, or estimate the amount on agarose gels.
10. Isolate the polyA^+ mRNA using Qiagen mRNA purification kit according to manufacturer's instructions.

3.5. *RL-SAGE Library Construction*

The detailed RL-SAGE protocol is available at the Plant-Microbe Interactome database (http://www.plant-microbe-interactome.org/wang). The major modified steps are described in the RL-SAGE method ***(6)***. The diagrammatic representation of the RL-SAGE procedure is shown in **Fig. 1**. The major steps in RL-SAGE library construction are described in this section.

1. Use approx 50 ng of mRNA for each RL-SAGE library procedure (*see* **Note 2**).
2. Equilibrate the oligo(dT) beads in lysis/binding buffer (*see* the Invitrogen I-SAGE kit instructions).
3. Synthesize complementary DNA (cDNA) using the reagents provided in the I-longSAGE kit (*see* the I-SAGE kit instructions).
4. Digest the cDNA with *Nla*III (*see* **Note 3**) and divide into two parts. Ligate pool A with adapter A and pool B with adapter B (**Fig. 1**; Adapter sequences have a *Mme*I binding site).
5. Release 21-bp tags from cDNA by digestion with *Mme*I (Type IIS restriction enzyme), and purify the tags from a 16% polycrylamide gel electrophoresis (PAGE; w/v).

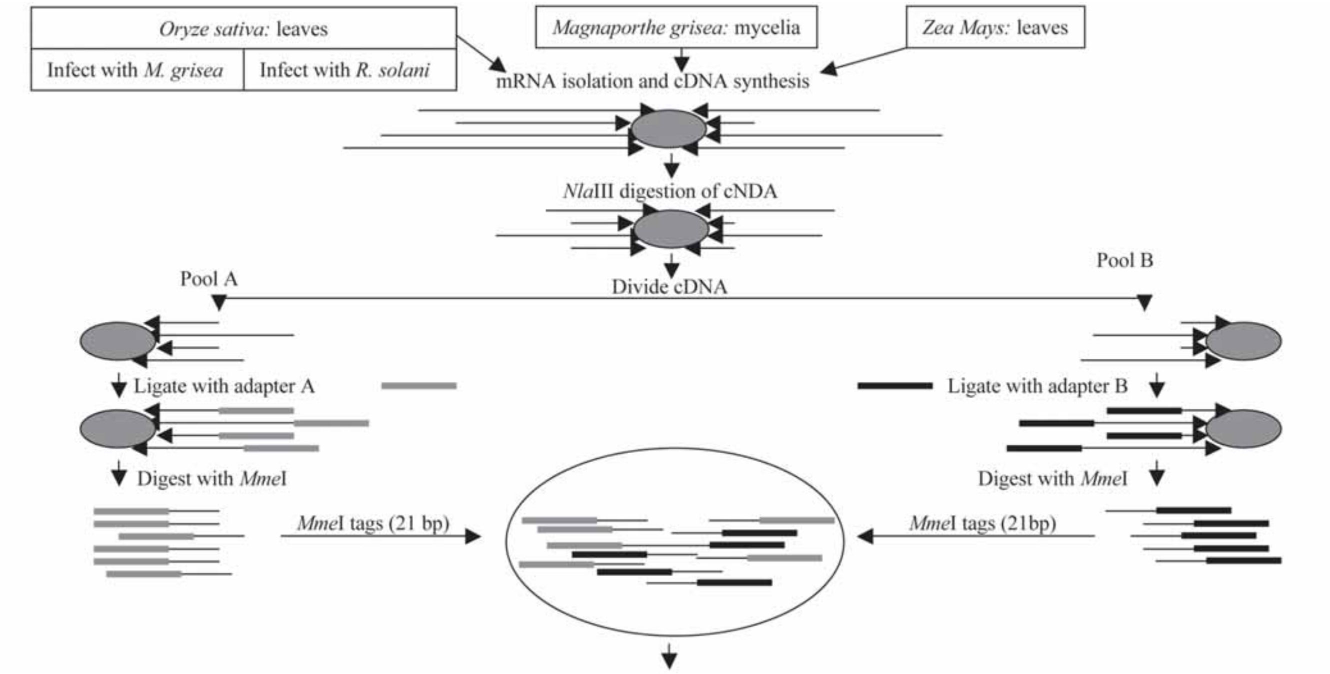

Fig. 1. Diagrammatic representation of robust-long serial analysis of gene expression (RL-SAGE) methodology and tag annotation. Total RNA from rice leaves, mycelium tissue of *M. grisea* and maize leaves (*see* **Sections 2** and **3**) is isolated. Messenger RNA (mRNA) is captured on magnetic beads containing oligo(dT)18 (shown as oval) Single- and double-stranded complementary DNA (cDNA) are synthesized according to the I-SAGE kit (Invitrogen) instructions. The cDNA with *Nla*III (recognition site CATG) is digested and only 3' fragments on the magnetic beads are retained. Then the cDNA is divided into two parts, pool A and pool B, which are ligated with adapters A and B, respectively. The ligated mix is digested with *Mme*I (type IIS restriction enzyme), which recognizes TCCGAC sequences in the adapter and cleaves 20 bases away in the cDNA sequence. The purified tags are self-ligated to

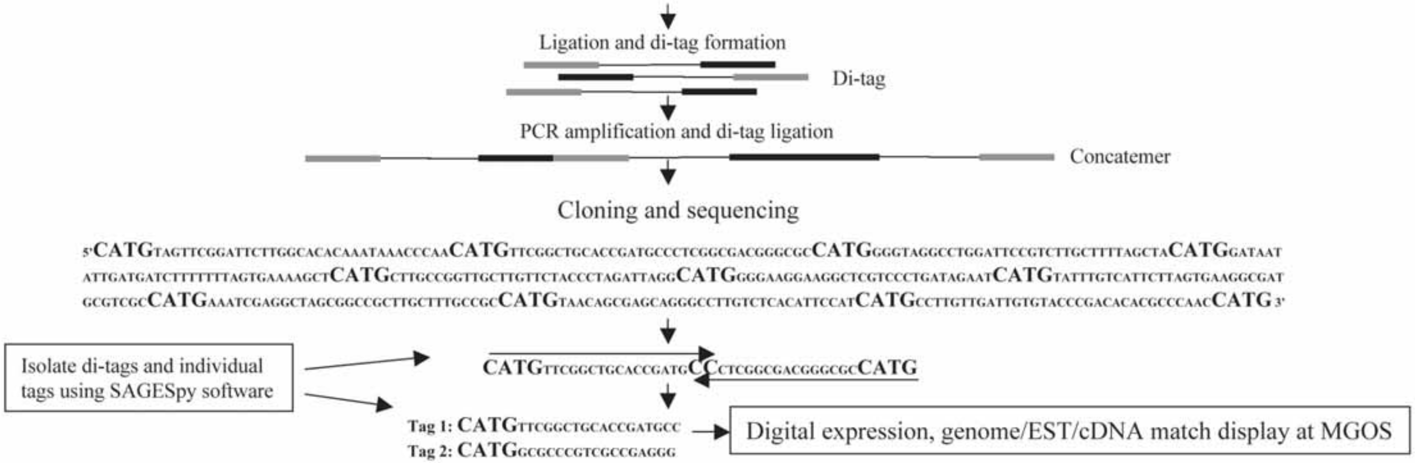

Fig. 1. (*continuted from previous page*) form di-tags. The linker sequences were removed from the polymerase chain reaction (PCR) amplified di-tags forgeneration of concatemers. The size-selected concatemers are cloned into the *Sph*I site of the pZERO-1 vector (Invitrogen) RL-SAGE clones are sequenced using M13 reverse and forward primers. SAGESpy software (*see* **Subheading 3.6.**, **Fig. 2**; Stahlbergh et al., unpublished) is then used to isolate di-tags and tags from the sequences. RL-SAGE tags are matched to genomic and EST sequences and the digital expression level of each tag is displayed on the *Magnaporthe grisea Oryza sativa* database (*see* **Subheading 3.7.**, **Fig. 3**).

6. Generate di-tags by overnight ligation of the tags from pool A and B.
7. Perform 20 to 50 di-tag PCRs (50-μL reactions; *see* **Note 4**).
8. Isolate the di-tag band (138 bp) on a 12% PAGE gel.
9. Remove linker sequences from di-tags by digesting with *Nla*III.
10. Purify the di-tag band (38 to 40 bp) on a 16% PAGE gel.
11. Purify di-tags again using Streptavidin magnetic beads.
12. Self-ligate di-tags to generate concatemers.
13. Perform a partial digestion of concatemers with *Nla*III (*see* **Note 5**).
14. Purify more than 500 bp concatemer bands on a 6% PAGE gel.
15. Prepare pZero-1 vector by *Sph*I digestion, and ligate concatemers.
16. Transform the ligation mix using TOP10 electrocompetent cells (Invitrogen).
17. Randomly pick 20 clones to check the size of inserts (*see* **Note 6**).
18. Pick 3000 to 7000 clones for sequencing.

3.6. SAGESpy Software for RL-SAGE Tag Annotation

SAGEspy is a set of high-performance applications employing the Cray Bioinformatics Library (CBL) and their Portable Cray Bioinformatics Library (PCBL) counterparts for conducting large-volume serial analysis of gene expression comparisons (*see* **Note 7**). The program was optimized for use on the Cray SV1 and Cray X1 systems employing OpenMP parallelism to speed the solution (http://www.osc.edu/research/bioinformatics/sagespy). **Figure 2** shows the major steps in RL-SAGE data processing using SAGESpy. The specific steps in the SAGE data analysis are described:

1. Get the sequences of RL-SAGE clones.
2. Isolate di-tags that are 40- to 42-bp long from the sequences of the RL-SAGE clones (**Fig. 1**)
3. Isolate individual RL-SAGE tags from the di-tags.
4. Convert the RL-SAGE tags into FASTA format.
5. Isolate virtual sense (from sense strand or Watson stand of the DNA) and antisense (or Crick strand of DNA) tags from a known nucleotide database (EST or genome sequence).
6. Convert all the virtual SAGE tags into FASTA format.
7. Use the SAGESpy software to match the experimental RL-SAGE tags (derived from the libraries of rice, *M. grisea*, or maize) with the virtual SAGE tags to calculate the matching rate of the experimental tags (**Fig. 1**).
8. Extract output files in FASTA format containing the list of tags, which hit target sequences and also list of tags with no hit in the database.
9. Identify novel transcripts, antisense transcripts, and alternatively spliced transcripts.
10. Repeat the matching analysis allowing one or two mismatches are allowed.

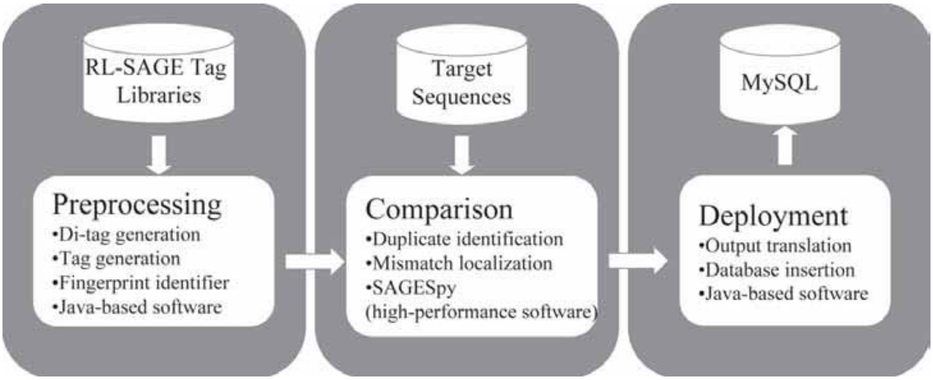

Fig. 2. Robust–long serial analysis of gene expression (RL-SAGE) tag libraries are preprocessed using Java-based programs for generating di-tags, and tags, and for assigning sequence fingerprint identifiers. The output is compared with target sequences (e.g., TIGR expressed sequence tags, KOME FL-cDNA, genomic DNA) using high-performance SAGESpy software, and an XML-based output file. The XML output file is translated and imported into the MySQL database for subsequent use for search and query support.

3.7. Digital Northern Analysis of RL-SAGE Tags at MGOS Database

The MGOS database (www.mgosdb.org) is a unique database that hosts EST and SAGE data of both rice and *M. grisea*. MGOS also contains the genome sequences of both rice and *M. grisea* (Soderlund et al., unpublished), which are displayed in the GMOD genome browser ***(18)***.

The RL-SAGE tags from the four rice libraries (OSJNGb, d, f, and g) and one *M. grisea* library (MG_SGa) are shown in **Fig. 3A**. Tags from each library are processed and displayed on the SAGE page of MGOS database (http://www.mgosdb.org/sage/). The browser contains tracks for the annotated genes and the RL-SAGE tags, along with other evidence, such as EST alignments. Annotation of RL-SAGE tags is as follows:

1. The tag hits an annotated gene, and hence inherits its UniProt ***(19)*** annotation.
2. It does not hit a gene, but it does hit the genome.

The flanking region is searched against UniProt, and if it hits a UniProt gene it uses that annotation. All unique tags are grouped together from all five libraries based on exact hits. These tags can be queried from the query page (**Fig. 3B,C**), which allows a versatile set of queries, such as the following:

1. Show all tags that have at least one or more occurrences from a selected subset of the libraries. This allows the user to view the shared tags across a set of libraries.

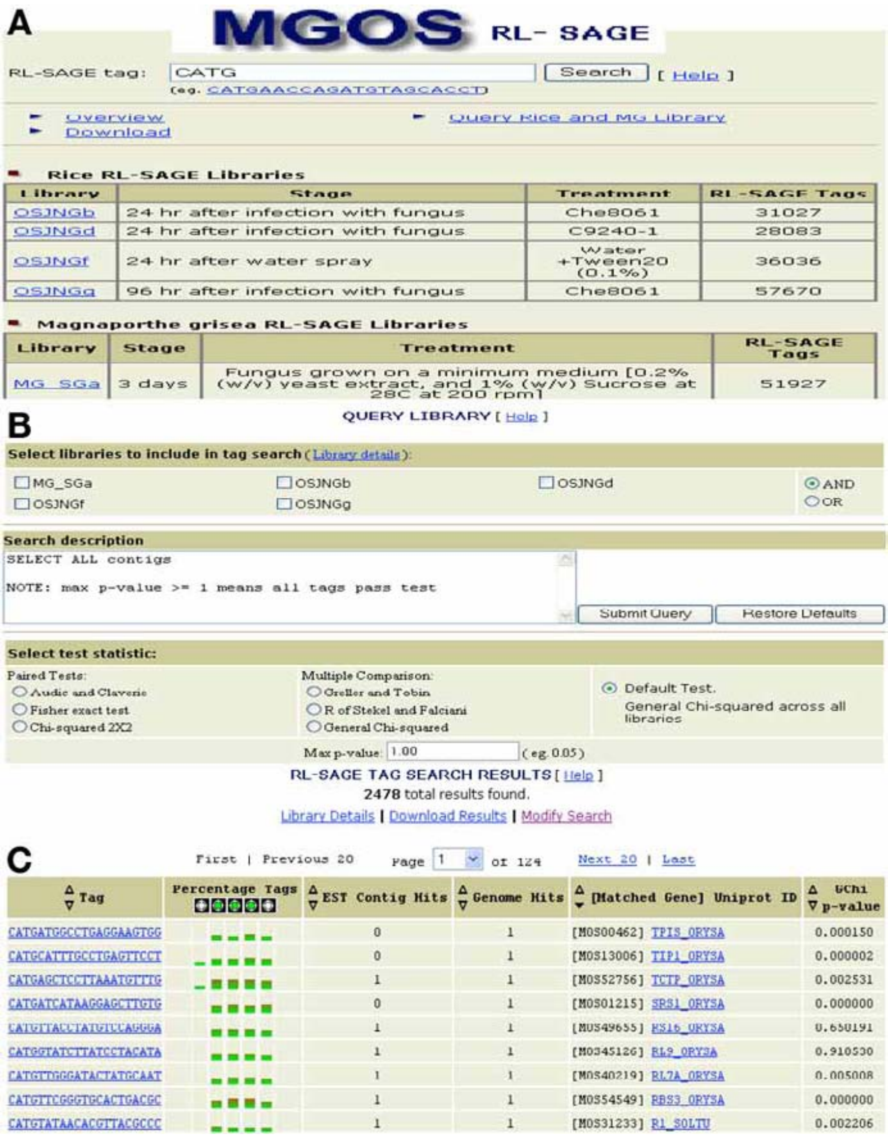

Fig. 3. The *Magnaporthe grisea Oryza sativa* database. **(A)** Features of four rice and one *M. grisea* robust–long serial analysis of gene expression (RL-SAGE) libraries are summarized. **(B)** The query page of the RL-SAGE libraries. The scrolling box shows exactly what will be searched for based on the filters set by the user. **(C)** Display of RL-SAGE search results. The top half of the table produced by the query and the second column shows the percentage levels of the five libraries. Scrolling one's computer mouse over any of the glyphs will show the exact number of tags in the library, and the data are normalized to tags per million.

2. Show all tags that have at least N or more occurrences in a selected subset of the libraries and do not have any tags from a different subset of the libraries. For example, the user may want to see the tags that are in both of the susceptible libraries but are not in the normal library.
3. Show the tags that have p-values less than N, where the *p* value is calculated based on one of the methods provided by IDEG6 (http://telethon.bio.unipd.it/bioinfo/IDEG6).
4. Show the tags that have one or more hits to the genome (or ESTs).
5. Show all tags that have a specific annotation. Any combination of the above.

A table of the tags that satisfy the query is displayed (**Fig. 3C**), where the columns of information shown are based on those selected by the user (**Fig. 3B**). For example, they may select the percentage of tags per library, UniProt matching ID, and overall quality of the tag (**Fig. 3B**). From the table, the user may select a specific tag, which then takes them to the tag's page, containing all of the information about it (**Fig. 3C**).

4. Notes

1. The RL-SAGE protocol was successfully used for library construction in rice, maize, and *M. grisea* in our laboratory. Two to three libraries could easily be constructed per month.
2. This method was optimized for a small amount of mRNA sample (approx 50 ng) for when only a limited amount of mRNA available for library construction is available.
3. Like in any other SAGE protocol, RL-SAGE also uses a single anchoring enzyme, *Nla*III (CATG), which may occur in every 256 bp. Therefore, a small portion (approx 3%) of transcripts in the genome might be uncovered during transcriptome analysis. To overcome this limitation, one could make another library using a different anchoring enzyme such as *Dpn*II.
4. Using this procedure, just 20 di-tag PCRs are sufficient to generate enough di-tags for concatenation
5. Partial digestion of concatemers with *Nla*III greatly improved the ligation efficiency of the concatemers (>150,000 clones from 10 μL of ligation mix) and increased the insert size (average of 1.0 kb). If all of the 150,000 clones are sequenced, 4.5 million tags can be recovered. Because of the high cost of sequencing, sequencing of 3000 to 5000 clones per library should be sufficient (100,000 to 200,000 individual tags).
6. Because of increased ligation efficiency, very few empty clones are found in the libraries. Therefore, clones can be picked randomly without using a PCR-screening strategy to remove empty clones. This improvement saves a lot of time when generating a RL-SAGE library. Following our method, two to three RL-SAGE libraries can be easily generated within a month.
7. SAGEspy software is available free to the users from the Ohio Supercomputer Center (http://www.osc.edu/research/bioinformatics/sagespy/).

Acknowledgments

The methods described here were developed with supports from a grant from the National Science Foundation's Plant Genome Research Program (no. 0115642). We are thankful to Rose Palumbo for her critical reading of the manuscript.

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14

Analysis of Gene Function in Rice Through Virus-Induced Gene Silencing

Xin Shun Ding, C. Srinivasa Rao, and Richard S. Nelson

Summary

Virus-induced gene silencing (VIGS) is a powerful RNA-silencing based technology adapted for the study of host-gene function. VIGS functions through the expression of a host gene from a virus vector. Both the virus-encoded host sequence and the homologous host target messenger RNA are destroyed or made inactive through a host surveillance system. Here, we describe procedures for the use of a new virus vector for VIGS in monocotyledonous hosts and, in particular, in rice (*Oryza sativa*), a species for which no VIGS vector was previously available.

Key Words: Virus-induced gene silencing; rice; monocotyledons; *Brome mosaic virus*; functional genomics; gene knockout.

1. Introduction

In the past few years, virus-induced gene silencing (VIGS) has been used to determine gene function in plants ***(1–4)***. The mechanism of VIGS is not fully understood; however, it is known to target RNA molecules in a sequence-specific manner. Although VIGS originally was a term used to describe the recovery of the host from virus infection, it now is used most often to describe the downregulation of host gene expression via a virus vector ***(2)***. Double-stranded RNA (dsRNA), produced by fold back of the single-stranded viral RNA or during virus replication through the activity of a viral RNA-dependent RNA polymerase, triggers silencing. The dsRNA is then cleaved into small interfering RNAs (siRNA; 21 to 25 nucleotides in length) by an RNase III-type dicer-like endonuclease. The resulting siRNAs are believed to provide a single strand of RNA for incorporation into the RNA-induced silencing complex. The RNA-induced silencing complex then identifies and cleaves additional single-

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

stranded target RNA sequences complementary to the "captured" siRNA sequence ***(2,5,6)***. Of interest, cleavage of additional target RNA through this pathway may not be the only method of controlling virus accumulation. It is possible that small RNAs derived from viral single-stranded RNA stem-loops, similar in structure to the microRNAs derived from host RNAs, inhibit virus accumulation through translational repression ***(4)***. With time, the mechanism of VIGS will be determined, but this lack of basic information does not prevent its practical use to study gene function. To date, multiple RNA plant virus vectors (e.g. *Tobacco mosaic virus*, *Potato virus X*, and *Tobacco rattle virus*) are available for VIGS in dicotyledonous plant species and one RNA virus vector, *Barley stripe mosaic virus*, is available for VIGS in two monocotyledonous species (barley and wheat ***[7–14]***). Here, we review the use of a plant virus vector newly created from a strain of *Brome mosaic virus* (BMV), to silence genes in various monocotyledons, including rice, through VIGS.

To construct this BMV vector, we isolated a virus from an infected Tall Fescue plant (*Festuca arundinacea* Schreb.) and purified viral RNA from the virus capsid ***(15,16)***. Previous analysis of the capsid, through electron microscopy, and viral RNA, through Northern blot analysis, indicated that this virus was a strain of BMV, which we named the fescue strain of BMV (F-BMV ***[15]***; Ding, X. S. and Nelson, R. S., to be submitted). Full-length reverse transcription polymerase chain reaction (RT-PCR) products of the three viral genomic RNAs were synthesized, gel purified, and ligated individually into the pGEM T-Easy vector (Promega, Madison, WI). The ligation products were transformed individually into *Escherichia coli* JM109-competent cells. The plasmids containing the viral complementary DNAs (cDNAs) were named pF1-11, pF2-2, and pF3-5 (for F-BMV cDNA representing BMV genomic RNAs 1, 2, and 3, respectively ***[15a]***). Three RNAs from the Russian strain of BMV (R-BMV ***[16]***) also were cloned (pB1-26 for RNA1, pB2-4 for RNA2, and pB3-3 for RNA3) using the same technique as described for the F-BMV. We determined that the viral sequence necessary for infecting rice did not reside in RNA 3 of BMV ***[15a]***). This information allowed us to use the clone representing RNA 3 from R-BMV, which has a more convenient restriction site for inserting host sequences than does the cDNA representing F-BMV RNA 3, in our VIGS vector. Co-inoculation of rice plants with transcripts from pF1-11, pF2-2, and pB3-3 harboring a fragment of phytoene desaturase from maize (*Zea mays*) caused silencing of this gene in rice ***(15)***.

VIGS has several advantages over other functional genomics approaches, such as no transformation of the host plant is required and the function of individual gene family members can be studied ***(2)***. An additional major advantage is the rapidity with which a researcher can observe a silencing phenotype during VIGS. A silencing phenotype can be observed within 3 wk after inserting a

host sequence into the BMV vector and inoculating plants. For important monocotyledonous crops such as rice and maize, for which no rapid gene knockout technology exists, VIGS with the F-BMV vector was effective in silencing genes ***(15a)***.

This chapter provides procedures to execute the following:

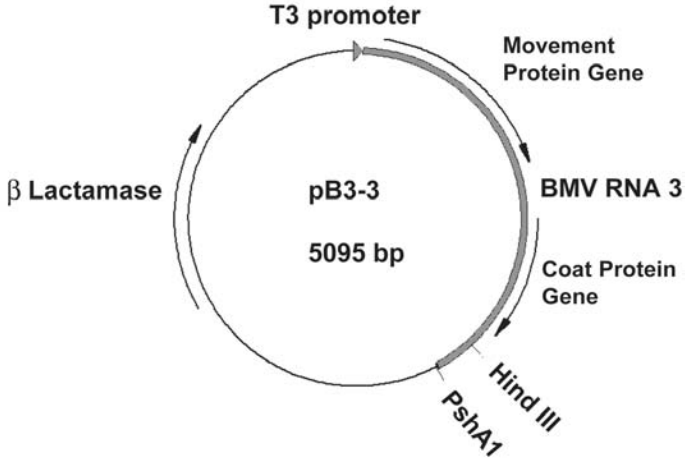
1. Insert plant sequences into bacterial plasmids and transfer them to the BMV vector.
2. Synthesize the BMV vector transcripts.
3. Inoculate plants with transcripts.
4. Analyze virus infection and gene silencing in the plants.

It is important that all plants inoculated with BMV or BMV harboring a foreign insert be kept inside a greenhouse or growth chambers. Infected plants and materials used to grow these plants (e.g., soil, pots, and trays) should be autoclaved before disposal to prevent release of the virus into the environment, as per regulations from the US Department of Agriculture governing the use of pathogens and transgenic viral sequences.

2. Materials

2.1. Insertion of Foreign Sequences Into the BMV Vector

1. pB3-3 (plasmid containing cDNA of R-BMV RNA 3; **Fig. 1**).
2. pGEM–T Easy vector (Promega; Madison, WI).
3. JM109 competent cells (10^8 cfu; Promega).
4. SOC medium: tryptone 2% (w/v), 8.6 m*M* NaCl, 2.5 m*M* KCl, 20 m*M* $MgSO_4$, 20 m*M* glucose (Sigma; St. Louis, MO).
5. 2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside): 20 mg in 1 mL of dimethylformamide (Promega)
6. 20% Isopropyl-β-D-thio-galactopyranoside (IPTG), 200 mg in 1 mL of H_2O (Promega)
7. Luria broth (LB) medium: 25 LB medium capsules per liter of distilled H_2O (Q-BIOgene; Irvine, CA).
8. LB agar medium: 16 LB agar medium capsules per 400 mL of distilled H_2O (Q-BIOgene).
9. LB liquid medium containing ampicillin (Sigma): autoclave LB liquid medium at 121°C for 20 min in 1-L autoclavable bottle. Remove the bottle from the autoclave after the pressure is fully released. Cool the medium to approx 50°C and add ampicillin (100 μg/mL medium; *see* **Note 1**). Mix well and store the medium at 4°C before use.
11. LB medium plate containing ampicillin (Sigma): autoclave LB agar medium at 121°C for 20 min. Remove the bottle from the autoclave after the pressure is fully released. Cool the medium to approx 50°C and add ampicillin (100 μg/mL medium). Mix well and pour the medium into 20 Petri dishes (100 × 15 mm; *see* **Note 1**). Store plates at 4°C before use.



	nucleotides
T3 RNA polymerase Promoter	16-33
BMV (*Brome mosaic virus*) RNA3	33-2135
BMV Movement Protein Gene	124-1018
BMV Coat Protein Gene	1275-1840
pGEM T Easy Vector	2141-5095
βLactamase (Ampicillin resistance) Gene	3401-4262

Fig. 1. Schematic drawing of pB3-3 showing restriction sites for inserting foreign deoxyribonucleic acid sequence or linearizing the plasmid before in vitro transcription.

12. LB medium plate containing ampicillin, X-gal and IPTG: Mix 20 μL of 2% X-gal solution and 30 μL sterilized H_2O with 50 μL of 20% IPTG. Spread the mix onto the surface of an LB medium plate containing ampicillin. Incubate the plate at 37°C for 1 h and then store the plate at 4°C before use (*see* **Note 1**).
13. T4 DNA ligase with 10X buffer (NEB; Ipswich, MA).
14. Nuclease-free water.
15. 1-kb DNA ladder (NEB).
16. *Hin*dIII restriction enzyme with 10X reaction buffer (NEB).
17. QIAquick PCR Purification kit (Qiagen; Valencia, CA).
18. QIAquick Gel Extraction kit (Qiagen).
19. Buffer P1 (Qiagen).
20. Buffer P2 (Qiagen).

2.2. Preparation of BMV RNA Transcripts and Inoculation of Plant

1. BMV clones (pF1-11, pF2-2, pB3-3, and pB3-3 containing a plant gene sequence).
2. Phenol/chloroform/IAA solution (Ambion; Austin, TX).
3. 3 *M* Sodium acetate, pH 5.2.
4. mMESSAGE mMACHINE T3 kit (Ambion).

5. *Spe*I restriction enzyme with 10X buffer (NEB).
6. *PshA*I restriction enzyme with 10X buffer (NEB).

2.3. Analysis of Virus Infection and Gene Silencing in Plants

1. Polyclonal antibody against BMV coat protein (*see* **Note 2**).
2. Primers to allow specific detection of host insert sequence in virus genome, virus genome, and host transcript targeted for silencing.
3. SuperScript reverse transcriptase with 5X first strand buffer and 0.1 *M* dithiothreitol (DTT; Invitrogen, Carlsbad, CA).
4. Taq DNA polymerase in buffer B with 10X PCR buffer and 25 m*M* $MgCl_2$ (Promega).
5. SUPERase In™ (Ambion).
6. 10 m*M* dNTP Mix: Mix 10 µL of 100 m*M* dATP, 10 µL of 100 m*M* dCTP, 10 µL of 100 m*M* dGTP, and 10 µL of 100 m*M* dTTP with 60 µL of nuclease-free H_2O.
7. Recombinant RNasin (40 U/µL; Promega).
8. TRIzol Reagent (Invitrogen).
9. DNaseI (RNase free; Ambion).
10. Chloroform.
11. Isopropanol.
12. Ethanol.
13. Agarose (Shelton Scientific, Shelton, CA).
14. Mortars and pestles: bake mortars and pestles at 180°C for 3 h before use.
15. 0.6-mL Microfuge tube (ISC Bioexpress, Kaysville, UT).

3. Methods

3.1. Insertion of Foreign Sequences Into the BMV Vector

To silence a plant gene of interest through VIGS, DNA representing a portion of the gene should be inserted into the *Hin*dIII restriction site within the pB3-3 construct (**Fig. 1**). Inserts can be 150 to 250 nucleotides in length for this vector. A gene fragment is amplified from total cellular RNA isolated from plant tissue through RT-PCR using primers specific for the gene. The amplified gene fragment is then ligated into the T-Easy vector. After purifying the T-Easy vector containing the inserted host-gene fragment, the fragment is released through digestion with *Hin*dIII and gel purified. The gene fragment then is ligated into the pB3-3 vector predigested with *Hin*dIII.

3.1.1. Synthesis and Ligation of a Target Host-Gene Fragment Into T-Easy Vector

1. Amplify a fragment (150 to 250 nucleotides in length) from a host mRNA representing a gene of interest using specific primers under predetermined RT-PCR conditions (*see* **Note 3**). An approx 100-µL PCR reaction mix is needed for each fragment.
2. Begin purifying the PCR product by mixing it with 500 µL of PB buffer (Qiagen; *see* **Note 4**).

3. Load the mixed solution onto a QIAquick spin column inserted into a 2-mL collection tube. Spin the column-collection tube unit at 15,000*g* for 1 min and discard the flow-through.
4. Add 0.7 mL of PE buffer (Qiagen) to the column and spin the column with a collection tube at 15,000*g* for 1 min. Discard the flow-through and spin the column with collection tube again at 15,000*g* for 1 min.
5. Place the column into a clean 1.7-mL microfuge tube. Add 50 μL of EB buffer (Qiagen) to the column and spin the column-collection tube unit at 15,000*g* for 1 min.
6. Concentrate the PCR product by spinning the tube in a microfuge under vacuum until the final volume of the PCR product is approx 5 μL.
7. Set up a ligation reaction by mixing 3 μL of PCR product (approx 200 ng of DNA) with 0.5 μL of pGEM-T Easy Vector (50 ng), 5 μL of 2X ligation buffer, 1 μL of nuclease-free H_2O, and 1 μL of T4 DNA ligase (Promega) in a microfuge tube.
8. Mix the contents by flicking the tube several times. Spin the tube briefly to collect the ligation mixture at the bottom of the tube and incubate the tube overnight at 4°C.

3.1.2. Transformation of Competent E. coli *Cells With Plasmid Containing the Host Sequence*

1. Place JM 109 competent cells from –80°C freezer on ice until just thawed (5 to 10 min).
2. Transfer 25 μL of just-thawed competent cells into a prechilled 14-mL Falcon tube (Becton Dickinson, Franklin Lakes, NJ). Add 2 μL of ligation mixture into the tube and mix gently by flicking the tube several times (*see* **Note 5**).
3. Incubate the tube on ice for 20 min and then subject the tube to a heat-shock treatment by holding it in a 42°C water bath for 40 s followed by a 2-min incubation on ice.
4. Add 250 μL of SOC medium into the tube and incubate the tube in a 37°C shaker set at 150 rpm for 1 h.
5. Plate 100 μL of transformed cell culture onto a LB medium plate containing ampicillin, X-gal and IPTG (*see* **Note 1**). If a greater number of colonies are desired, spin the transformed competent cell culture at 1000*g* for 2 min, resuspend the pellet in 100 μL of fresh LB liquid medium, and plate the cells on one LB medium plate containing ampicillin, X-gal, and IPTG.
6. Incubate the plate overnight in a 37°C incubator. After incubation, pick two to three white colonies from the plate with toothpicks and inoculate each colony to individual Falcon tubes containing 2.5 mL of LB liquid medium with ampicillin.
7. Incubate the Falcon tubes in a 37°C shaker set at 250 rpm overnight.

3.1.3. Isolation of T-Easy Vector With Plant-Gene Insert

1. Before isolating plasmid DNA from the overnight cell culture, take 0.5 mL of the overnight culture and mix with 0.5 mL of sterile 50% glycerol in a sterile 1.7-mL microfuge tube. Store the tube at –70°C for future use.

2. Transfer 1.7 mL of the remaining overnight cell culture into a microfuge tube. Pellet cells by centrifuging the microfuge tube at 15,000*g* in a microfuge at room temperature (RT) for 5 min (*see* **Note 6**).
3. Discard the supernatant. Resuspend the pellet in 0.3 mL of buffer P1 with RNase A (Qiagen) by pipetting the cells up and down until the pellet is fully dissolved. Incubate the microfuge tube at RT for 5 min.
4. Add 0.3 mL of lysis buffer P2 (Qiagen) into the microfuge tube and mix by inversion five or six times. Incubate the tube on ice for 5 min.
5. Add 0.3 mL of 3 *M* potassium acetate, pH 5.5, to the microfuge tube and mix by inversion two to three times. Incubate the microfuge tube on ice for 10 min.
6. At RT, centrifuge the mixture at 15,000*g* for 10 min in a microfuge. Pour the supernatant into a clean microfuge tube and centrifuge again at 15,000*g* for 5 min.
7. Pour the supernatant (approx 0.9 mL) into a clean microfuge tube. Add 0.65 mL of isopropanol into the microfuge tube, mix and incubate the mixture on ice for 20 min.
8. At RT, centrifuge the mixture at 15,000*g* for 15 min to pellet plasmid DNA. Discard the supernatant. To wash the DNA, carefully add 0.7 mL of 75% ethanol to the tube, flicking the tube several times, and centrifuge the tube at 15,000*g* for 5 min.
9. Carefully remove the supernatant without dislodging the DNA pellet. Dry the remaining pellet by centrifugation in a vacuum centrifuge (e.g., Savant SC110A, Cambridge Scientific Products, Cambridge, MA) for 5 min.
10. Resuspend the pellet in 20 μL of nuclease-free H_2O. Estimate the concentration of the plasmid DNA preparation by electrophoresis in a 1% agarose gel containing 0.1 μg of ethidium bromide per milliliter of agarose solution (*see* **Note 7**) and comparison with differing known concentrations of 1-kb DNA ladder loaded in adjacent lanes. Alternatively, determine the plasmid concentration with a spectrophotometer.

3.1.4. Transfer of Plant-Gene Sequence From pGEM T-Easy to pB3-3

1. To release the plant-gene sequence from the T-Easy plasmid, mix 5 μL of the purified T-Easy plasmid containing the plant sequence (approx 2 μg of DNA) with 3 μL of 10X *Hin*dIII reaction buffer (NEB), 21 μL of nuclease-free H_2O and 1.5 μL of *Hin*dIII restriction enzyme (NEB) in a microfuge tube. Incubate the tube at 37°C for 2 h.
2. After digestion, mix the DNA sample with a loading dye and load the sample onto a 1% agarose gel containing 0.1 μg of ethidium bromide per milliliter of agarose solution. Electrophorese to separate the DNA fragment containing the plant gene sequence from the T-Easy vector DNA. Also load a DNA ladder as a size marker.
3. Visualize DNA bands with an ultraviolet transilluminator. Excise the gel fragment containing the plant gene sequence using a clean razor blade and transfer the gel slice into a microfuge tube (approx 300 μg of gel slice per tube).
4. Add 1 mL of QG buffer (Qiagen; *see* **Note 8**) to the microfuge tube and incubate at 50°C for 10 min. Invert the tube several times during the incubation. The gel slice should be completely dissolved before moving to the next step.

5. Place a QIAquick spin column into a 2-mL collection tube. Add 0.75 mL of dissolved gel solution into the column. Centrifuge the column and collection tube unit in a microfuge at 15,000*g* and RT for 1 min.
6. Discard the flow-through and place the column back onto the same collection tube. Add the remaining gel solution onto the column and then centrifuge the column-collection tube unit at 15,000*g* and RT for 1 min.
7. Discard the flow-through, re-attach the collection tube, and add 0.75 mL of fresh QG buffer to the column. Centrifuge the column-collection tube unit at 15,000*g* and RT for 1 min.
8. Wash the column by adding 0.75 mL of PE buffer (Qiagen; *see* **Note 8**) and centrifuge the column and collection tube at 15,000*g* for 1 min. Discard the flow-through and spin the column-collection tube unit again at 15,000*g* for an additional minute, discarding the flow-through.
9. Place the column above a clean 1.7-mL microfuge tube. Add 50 μL of nucleotide-free H_2O or EB Buffer (Qiagen; *see* **Note 8**) to the column and centrifuge the column-collection tube unit at 15,000*g* for 1 min. Concentrate the purified DNA fragment by centrifuging the collection tube under vacuum for 20 min or until the final volume of the DNA sample is approx 5 μL.
10. To ligate the purified DNA fragment containing the host gene sequence into pB3-3, set up a ligation reaction by mixing 3 μL of the gel-purified DNA fragment (approx 200 ng), 1 μL of pB3-3 vector linearized with *Hin*dIII enzyme (approx 100 ng), 5 μL of 2X ligation buffer, and 1 μL of T4 DNA ligase (Promega) in a 0.7-mL microfuge tube.
11. Mix the reagents by flicking the tube several times. Centrifuge the tube briefly to collect the reaction mixture in the bottom of the tube and incubate overnight at 4°C.
12. Transform JM109 competent cells with the ligation product and isolate pB3-3 containing the plant gene sequence from the transformed cells as described in the **Subheading 3.1.2.**

3.2. Synthesis of BMV RNA Transcripts and Plant Inoculation

Before in vitro transcription, the three plasmids containing the BMV genome sequences should be linearized using *Spe* I (pF1-11) or *PshA* I (pF2-2 and pB3-3) restriction enzymes. RNA transcripts representing the three BMV genomic RNAs are prepared individually from the linearized plasmids in vitro and the products mixed before inoculating host plants. RNA transcripts can be stored at –70°C before use. All reagents and materials used during the in vitro transcription reactions should be nuclease-free.

3.2.1. Synthesis of RNA Transcripts

1. Linearize pF1-11, pF2-2, and pB3-3 individually in 50-μL reactions containing 2 μg of template DNA, 5 μL of 10X the restriction enzyme buffer, 0.5 μL of bovine serum albumin (100 mg/mL), and 1.5 μL of *Spe* I (pF1-11) or 1.5 μL of

PshA I (pF2-2, pB3-3, and pB3-3 with insert) restriction enzymes. Incubate the reactions at 37°C for 1.5 h.

2. After incubation, add 50 μL of a phenol/chloroform/IAA solution to each microfuge tube (*see* **Note 9**). Mix the content thoroughly by vortexing the microfuge tubes for 20 s and then centrifuge at 15,000*g* and RT for 5 min.
3. Transfer the upper liquid phase (approx 50 μL) from each tube into a clean nuclease-free microfuge tube.
4. Add 50 μL of chloroform to each microfuge tube. Mix the contents thoroughly by vortexing the microfuge tubes for 15 s and then centrifuging the tubes at 15,000*g* for 5 min.
5. Transfer the upper liquid phase (approx 50 μL) from each tube into a clean nuclease-free microfuge tube. Add 5 μL of 3 *M* sodium acetate, pH 5.2, and 100 μL of ice-cold ethanol to each tube. Mix the content by flicking the tubes several times. Incubate the tubes at –20°C for 1 h or at –70°C for 20 min.
6. Centrifuge the microfuge tubes at 15,000*g* for 15 min and discard the supernatant.
7. Add 0.7 mL of ice-cold 70% ethanol to each tube. Flick the tubes several times and centrifuge them at 15,000*g* and RT for 5 min.
8. Discard the supernatant from each tube. Dry the pellets in a vacuum centrifuge for 5 min.
9. Resuspend each pellet in 15 μL of nuclease-free H_2O. Visualize the efficiency of the linearization and estimate the concentration of each linearized template by electrophoresis through a 1% agarose gel containing 0.1 μg of ethidium bromide per milliliter of agarose solution. An aliquot of 1-kb DNA ladder containing a fragment of known concentration should be loaded on the gel to aid in determining the amount of product produced and the efficiency of the reaction.

   Transcripts from the individual linearized templates are synthesized by adding 10 μL of 2X NTP/CAP solution, 2 μL of 10X reaction buffer (both from mMessage mMachine kit, Ambion) and 6 μL of template DNA (approx 0.5 μg, representing BMV RNA 1, 2, or 3) into a nuclease-free microfuge tube.
10. The in vitro transcription reaction is initiated by adding 2 μL of T3 enzyme mix into each tube, mixing well by flicking the tubes several times and incubating tubes at 37°C for 1.5 h.
11. The product RNA transcripts can be used to infect a plant immediately, or stored at –70°C for later use.

3.2.2. Inoculation of Plant With Transcript

BMV RNA transcripts can be used to infect seedlings directly through mechanical inoculation. To achieve more successful infections, the viral transcripts should be inoculated first to *Nicotiana benthamiana* seedlings; this plant is easily infected and allows high titers of many viruses to accumulate in its leaves. Crude extracts from *N. benthamiana* leaves are then used to infect rice seedlings.

1. Seeds of *N. benthamiana* are sown in a soil mixture (Metromix 350; SunGro Horticulture, Bellevue, WA) in small pots. At 3 wk after planting, individual

seedlings are transplanted into 4.5-in. pots. Seeds of rice are planted directly into a soil in 4.5-in. pots. Rice is particularly intolerant of suboptimal soil conditions. Greenhouse temperatures for *N. benthamiana* and rice are 25/20°C (day/night). Seedlings are watered and fertilized as needed.

2. Mix in vitro transcripts representing BMV RNAs 1, 2, and RNA 3, harboring or not harboring a host-gene insert. Select two similar sized *N. benthamiana* seedlings at 5 d after transplanting. Dust two leaves of each plant with Carborundum (320 grit; Sigma) through cheesecloth layers and inoculate the leaves by gently rubbing 7.5 µL of mixed transcripts across the surface of each leaf. The inoculated plants are then moved to a growth chamber at 24/20°C (day/night). As controls, leaves of two *N. benthamiana* seedlings are inoculated with mixed transcripts representing BMV RNAs 1, 2, and 3, without the host-gene insert.
3. Monitor virus levels and stability of the plant target sequence in the virus in each inoculated *N. benthamiana* plant by immunocapture RT-PCR (IC) using primers specific for BMV RNA3 sequences as described in **Heading 3.**
4. Harvest leaf tissue from infected *N. benthamiana* plants, grind the tissue in 0.1 *M* phosphate buffer, pH 6.5 (1:10, w/v) at 4°C.
5. Dust leaves of 2-wk-old rice seedlings with Carborundum and inoculate them with the crude extracts prepared from the infected *N. benthamiana* leaves in **step 4**. The inoculated rice seedlings are grown in a greenhouse at 25/20°C (day/night) and monitored for virus infection and gene silencing through symptom observation, IC RT-PCR and semiquantitative RT-PCR described in **Heading 3.**

3.3. Analysis of Virus Infection and Gene Silencing in Plant

The progress of infection by the BMV vector in the host can be monitored through IC RT-PCR. The stability of the plant-gene insert in the virus vector during systemic infection of the host can also be monitored using this technique.

3.3.1. Detection of Virus Through IC RT-PCR

This procedure involves the capture of BMV virions from crude plant extracts on walls of microfuge tubes precoated with an antibody specific for the BMV coat protein (CP). Reverse transcription reactions can then be performed in the same microfuge tube without the need to release viral RNA from the bound virions. The resulting first strand cDNAs are then amplified via PCR using primers specific to the BMV RNA 3 sequence.

1. Dilute 1 µL of BMV CP antibody ***(15)*** in 2.5 mL of coating buffer (coating buffer: 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ to 1 L distilled H_2O. Adjust pH to 9.6 with HCl).
2. Add 30 µL of diluted antibody solution to each 0.6-mL microfuge tube (ISC Bioexpress) and incubate the tubes overnight at 4°C.
3. Wash each tube twice using 0.1 *M* phosphate buffer (PB), pH 7.0. After removing PB from the tubes, store them at –20°C for later use.

4. Collect two leaf discs by pressing leaf with the lid of a microfuge tube (approx 100 mg total) from each plant and grind them inside the microfuge tube in 100 µL of 0.1 *M* PB with disposable plastic pestles at RT.
5. Add 30 µL of crude leaf extract from each sample to a 0.6-mL tube pre-coated with the CP antibody and incubate the tube overnight at 4°C.
6. Wash each tube three times with 0.1 *M* PB.
7. Add 5 µL of solution containing a primer complementary to the 3' end of the BMV RNA 3 sequence (0.5 µL of 10 m*M* reverse primer in 4.5 µL of DEPC H_2O) to each tube and incubate the tubes at 70°C for 10 min followed by 2 min on ice.
8. Add 5.0 µL of premixed RT reagents (2 µL of 5X first-strand buffer, 1 µL of 0.1 *M* DTT, 0.5 µL of 10 m*M* dNTP Mix, 0.5 µL of RNasin, 0.5 µL of Super Script RT, and 0.5 µL of nuclease-free H_2O) to each tube and incubate at 42°C for 1 h.
9. After 1 h, add 1 to 2 µL of RT reaction mix to a PCR tube containing 18.5 µL of mixed PCR reagents (2 µL of 10X PCR buffer, 2 µL of 25 m*M* MgCl, 0.5 µL of 10 µ*M* forward primer (specific for the BMV CP gene sequence), 0.5 µL of 10 µ*M* reverse primer, 0.4 µL of 10 m*M* dNTPs, 0.1 µL of Taq polymerase, 14.5 µL of nuclease-free H_2O).
10. Perform 30 cycles of PCR under predetermined conditions.
11. Analyze PCR products on a 1% agarose gel containing 0.1 µg of ethidium bromide per milliliter of agarose solution. Some quantitation of virus accumulation can be achieved by decreasing the number of PCR cycles so the system is not saturated. Products from BMV vectors maintaining the host-gene insert should yield a higher-molecular-weight fragment than that observed from the BMV vector with no host insert (**Fig. 2**).

3.3.2. Analysis of Target-Gene Silencing Through Semiquantitative RT-PCR

The level of plant-target-transcript silencing can be monitored through semiquantitative RT-PCR. The procedure described in this section uses primers specific for the target gene, complementary or identical to sequences outside the region of the gene expressed within the virus vector, and the gene encoding elongation factor-1α, as an internal control (other host mRNAs may serve as internal controls; the best are those whose levels do not fluctuate during virus infection). Relative transcript levels for the gene of interest between various treatments are estimated by comparing the intensity of PCR product gel bands obtained after multiple PCR cycle numbers and normalizing the intensities according to estimates of the substrate RNA levels based on results from the internal control.

3.3.2.1. Isolation of Total RNA From Leaf Tissues

1. Harvest leaf tissue from plants infected with the BMV vector containing or not containing the plant gene insert at 2 to 3 wk after inoculation. The harvested tissue can be used for RNA isolation immediately or stored at –70°C for future use.

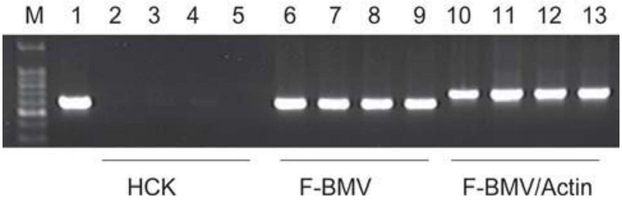


Fig. 2. Presence of virus and plant-gene insert in inoculated plants determined by immunocapture reverse-transcription polymerase chain reaction. *Brome mosaic virus* (BMV)-based virus-induced gene silencing (VIGS) vectors containing or not containing an action gene insert (F-BMV/Actin and F-BMV, respectively) were inoculated to rice plants. Four weeks after inoculation, BMV RNA3-specific primers flanking the actin insert site were used to determine the presence of BMV and actin insert. Templates for polymerase chain reaction were lane 1, purified BMV plasmid vector (pB3-3); lanes 2 through 5, extract for uninoculated plants (HCK); lanes 6 through 9, extract from plants inoculated with vector (F-BMV); lanes 10 to 13, extract from plants inoculated with vector carrying the actin insert (F-BMV/Actin). Each lane represents the analysis of a single plant. M, marker lane.

2. Take 0.1 g of tissue from each sample, add liquid nitrogen, and grind using a mortar and pestle. Add 1 mL of TRIZOL reagent to each mortar while the tissue is still frozen and continue grinding for 1 min (*see* **Note 10**). Transfer the crude sap into cold 1.7-mL microfuge tubes and incubate the tubes at RT for 5 min.
3. Add 0.2 mL of chloroform to each microfuge tube. Invert tubes several times to mix the contents and incubate at RT for 5 min.
4. Centrifuge microfuge tubes at 15,000*g* for 15 min at 4°C.
5. Transfer the upper aqueous phase containing RNA to individual nuclease-free microfuge tubes. Add 0.5 mL of isopropanol into the aqueous phase in each tube. Mix thoroughly by inverting tubes several times. Incubate the tubes at RT for 10 min.
6. Centrifuge the microfuge tubes at 15,000*g* for 10 min at 4°C.
7. Discard the supernatant. Add 0.7 mL of 75% ethanol to each microfuge tube. Flick the tubes several times and then centrifuge them at 15,000*g* for 5 min at 4°C.
8. Discard the supernatant. Dry the RNA pellets by vacuum centrifugation for 5 min at RT.
9. Dissolve each pellet in 30 μL of nuclease-free water.
10. Determine the RNA concentration for each sample by diluting 1 μL of isolated RNA in 500 μL of nuclease-free H_2O and measuring its absorbance (A) value at 260 and 280 nm in a UV spectrophotometer. Acceptable A260/A280 ratios for RNA are between 1.6 and 1.8. An A value of 1 at A_{260} and measured in a cuvet with a 1-cm path length approximates 40 μg/mL of RNA.
11. Add 1 μL of RNase-free DNase I to each RNA sample and incubate the samples at 37°C for 15 min to remove contaminating DNA.

3.3.2.2 Reverse Transcription

1. Add 10.5 μL of total RNA (2 μg), 1 μL of 10 μ*M* Oligo(dT) primer, and l μL of dNTP Mix (10 m*M* each) into a nuclease-free microfuge tube.
2. Incubate the tube at 70°C for 5 min and then on ice for 2 min.
3. Add 4 μL of 5X first-strand buffer, 2 μL of 0.1 *M* DTT, and 0.5 μL of SUPERase In™ to the tubes. Mix the content thoroughly by flicking the microfuge tube several times, centrifuge to collect solution in base of tube and incubate the tube at 37°C for 2 min.
4. Add 1 μL of SuperScript™ II Reverse transcriptase to each tube and flick the tube several times to mix the content. Centrifuge to collect solution in base of tube.
5. Incubate the microfuge tube at 37°C for 1 h. Inactivate the reaction by heating the tube at 70°C for 15 min. The resulting cDNA is the template for amplification during the subsequent PCR.

3.3.2.3. Polymerase Chain Reaction

1. For each sample, mix 4 μL of cDNA obtained in **Subheading 3.3.2.2.**, **step 5** above with 53.6 μL of nuclease-free water, 8 μL of 10X PCR buffer, 8 μL of 25 m*M* $MgCl_2$, 2 μL of 10 m*M* dNTP mix, 2 μL of forward primer (10 μ*M*), 2 μL of reverse primer (10 μ*M*) and 0.4 μL of *Taq* DNA polymerase (5 U/μL). Aliquot the 80 μL of reaction mixture equally into 4 PCR tubes.
2. Perform PCR under predetermined conditions. Remove one tube from the thermocycler at 20, 25, 30, and 35 cycles of reaction, respectively.
3. Visualize PCR products by electrophoresis in a 1.5% agarose gel containing 0.1 μg of ethidium bromide per milliliter of agarose solution. Determine the silencing result by comparing the band intensity of PCR products obtained after various PCR cycle numbers (**Fig. 3**). RNA samples from plants infected with BMV not expressing a plant gene sequence should be included in the experiment as a control. RNA transcript levels from host genes that are minimally affected by virus infection (e.g., elongation factor-1α, ubiquitin, or actin) should be included in the same experiment as controls to normalize results based on template levels.

4. Notes

1. Ampicillin can be first dissolved in sterilized H_2O (100 mg/mL H_2O) and stored at –20°C (stable for at least 2 mo). Addition of ampicillin to LB medium and preparation of LB medium plates is best done in a sterile laminar flow hood to avoid contamination. LB-agar medium made previously and stored at 4°C can be melted in a microwave set on "defrost" before being poured into Petri dishes. The solidified poured plates can be placed inside plastic bags and stored at 4°C for approx 1 mo.
2. Polyclonal antibody against BMV coat protein was prepared by injecting a rabbit with purified BMV virion ***(15)***. The antibody was mixed (1:1, v/v) with 50% sterilized glycerol and stored at –20°C. The antibody solution also contains 0.02%

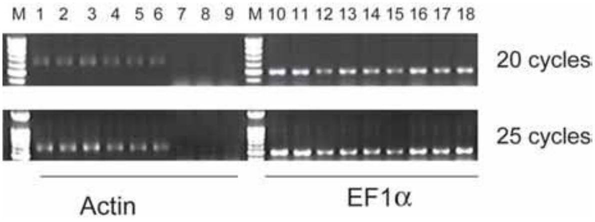


Fig. 3. Actin transcript downregulation during virus-induced gene silencing (VIGS) determined by semiquantitative reverse-transcription polymerase chain reaction. RNA extracted from rice plants inoculated with *Brome mosaic virus* (BMV)-based VIGS vectors containing or not containing an action gene insert (F-BMV/Actin and F-BMV, respectively) was analyzed for actin and elogation factor (EF)-1α transcript levels to determine the effectiveness of VIGS on actin messenger RNA expression. Primers specific for endogenous actin (550 bp; lanes 1–9) and EF-1α (400 bp; lanes 10–18) gene were used to assess the host-encoded transcript levels during infection. Transcript levels were assessed after 20 and 25 cycles of polymerase chain reaction. To avoid signal from actin sequence expressed from F-BMV/Actin, the actin primers were specific for regions of the transcript flanking the actin sequence in the virus vector. Templates for reverse-transcription polymerase chain reaction were lanes 1–3 and 10–12, RNA from healthy plants; lanes 4–6 and 13–15, RNA from plants inoculated with F-BMV; lanes 7–9 and 16–18, RNA from plants inoculated with F-BMV/Actin. Each lane represents the analysis of a single plant. M, marker lane.

sodium azide (a chemical harmful to humans, but present as an antibiotic). Wear disposable gloves while working with solutions containing sodium azide.

3. A DNA fragment of 150 to 250 nucleotides should be amplified from a gene of interest through RT-PCR. Both forward and reverse primers should be designed based on the sequence of the gene. It is advantageous to compare sequences to be used for primers with other sequences from plants, both from the target gene and other plant sequences, to verify that they are unique (i.e., to avoid off-site targeting). Each primer should contain a *Hin*dIII restriction site at its 5' end so that the fragment can later be released from the T-Easy vector and inserted into the *Hin*dIII site within the BMV vector.
4. PB buffer contains chemicals harmful to human. Always wear a laboratory coat, disposable gloves, and eye protection while working with this solution.
5. In our experiments, we use 25 μL of competent cell suspension per transformation. The remaining competent cell suspension should be returned immediately to the –80°C freezer for future use. Repeated freeze–thawing decreases the competency of these cells for transformation, so it would be wise to aliquot cells into small volumes in separate tubes. The used pipet tips and Falcon tubes should be placed inside a biohazard bag for autoclaving. Wear disposable gloves while performing the transformation.

6. SDS is harmful to humans. Wear disposable gloves and eye protection while weighing out SDS and isolating plasmid DNA from the overnight cell culture. The used culture medium should be killed with Clorox. All used Falcon and microfuge tubes and pipet tips should be placed inside a biohazard bag for autoclaving.
7. Ethidium bromide may cause hereditable damage to humans. Always wear gloves, eye protection, and laboratory coat when running gels. TBE buffer and agarose gels containing ethidium bromide should be collected in waste containers and disposed of following proper procedures.
8. QG buffer contains chemicals harmful to humans. Always wear a laboratory coat, disposable gloves, and eye protection while working with this solution. Residual ethanol from PE buffer can affect later digestions and ligations. It can be removed completely from the column by the second centrifugation (for 1 min). When using water to elute DNA, make sure that the water pH value is between 7.0 and 8.5.
9. Phenol and chloroform are extremely toxic. Always wear a laboratory coat, disposable gloves, and eye protection while working with these chemicals.
10. TRIzol is toxic and in contact with skin. Always wear a laboratory coat, disposable gloves, and eye protection while working with it.

Acknowlegments

The authors wish to thank Drs. Xian Zhi He and Justin Pita and Kim Ballard for helpful comments on the manuscript.

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15

Use of RNA Interference to Dissect Defense-Signaling Pathways in Rice

Chuansheng Mei, Xiangjun Zhou, and Yinong Yang

Summary

The RNA inference (RNAi) technique is a powerful tool to suppress gene expression and has been widely used for functional discovery of eukaryotic genes. To dissect defense-signaling pathways in rice, it is important to generate a series of rice mutant lines deficient in or insensitive to major signal molecules such as jasmonic acid and ethylene. Here we describe an RNAi protocol for generating and characterizing transgenic gene-silencing lines defective in rice jasmonic acid signaling. The RNAi technique should be useful for effective suppression of host genes encoding signaling components and facilitating the dissection of defense signal pathways in rice.

Key Words: Rice; RNA interference; gene silencing; defense signaling.

1. Introduction

RNA interference (RNAi) causes gene-specific silencing based on sequence homology-dependent degradation of cognate messenger RNA (mRNA ***[1,2]***). The phenomenon, also known as posttranscriptional gene silencing, was first discovered in petunia, in which overexpression of the *CHS* gene encoding a key enzyme (chalcone synthase) in anthocyanin biosynthesis surprisingly resulted in downregulation of anthocyanin levels ***(3,4)***. Recent studies show that RNAi is mediated by short-interfering RNAs or microRNAs, which result from the cleavage of a double-stranded RNA by an RNase III-related nuclease Dicer ***(5)***. RNAi is universally present in plants, animals, and fungi and is now considered an important mechanism for endogenous gene regulation, development, and host defense. Furthermore, the gene-silencing method based on RNAi recently has emerged as a powerful tool for the functional discovery of eukaryotic genes and genetic engineering of host resistance against viral infection ***(5,6)***.

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

In comparison with transfer DNA or transposon insertion, chemical or radiation treatment, and other mutagenesis approaches, there are a number of advantages for using RNAi to generate loss-of-function (knockout or knockdown) mutants, especially in a plant species with a large-size genome. First, RNAi allows targeted and effective knockout or knockdown of specific genes at a high frequency without random and laborious screening of loss-of-function mutants from large mutant populations. Second, simultaneous suppression of redundant or homologous genes (e.g., multiple members of a same gene family) can be achieved with RNAi ***(7)***. Third, inducible RNAi may provide an effective way for functional analysis of genes whose mutation will lead to embryonic or early developmental lethality ***(8)***. Furthermore, a large population of gene-silencing lines can be generated using high-throughput RNAi ***(9,10)***, which will complement other mutagenesis approaches for both forward and reverse genetics-based functional genomic studies.

Stable gene-silencing lines can be generated via plant transformation using various RNAi constructs that may include sense, antisense, inverted repeat, or tandem inverted repeat of specific genes. Chuang and Meyerowitz ***(11)*** first reported the effective gene silencing in *Arabidopsis* using an RNAi construct composed of an inverted repeat of the gene of interest. Smith et al. ***(12)*** proposed including an intron for more effective gene silencing based on the hypothesis that excision of the intron might improve alignment of the complementary sequences flanking the intron. By incorporating a chemical-inducible promoter, Guo et al. ***(8)*** constructed an inducible RNAi vector that should be advantageous to the study of lethal genes required for embryo or early development. For high efficient, large-scale gene silencing, Wesley et al. ***(9)*** developed a high-throughput RNAi vector (pHELLSGATE) by combining with Invitrogen's Gateway recombination technology. With slight modifications, a similar vector (pANDA) was constructed for high-throughput RNAi in rice plants ***(13)***. In addition, Brummell et al. ***(10)*** developed an innovative method for high-throughput generation of specific RNAi contructs by one-step, simple cloning of any target gene fragment between a *35S* promoter and an inverted repeat of a heterologous 3' untranslated region. As a result, a variety of RNAi vectors for different purposes are now available for generating stable gene silencing lines.

During the past decade, significant progress has been made toward the understanding of defense signaling in *Arabidopsis*, a model dicot. However, little is known about signal transduction and defense pathway interactions leading to host-defense response in rice, a model monocot and economically important food crop. Our laboratory has previously used RNAi to knockout/knockdown rice *OsMAPK5* gene encoding a stress-responsive mitogen-activated protein kinase and successfully demonstrated the importance of *OsMAPK5* in rice biotic and abiotic signal transduction ***(14)***. We also have generated by

RNAi a series of transgenic rice lines deficient of or insensitive to major defense signal molecules such as jasmonic acid (JA) and ethylene. These transgenic RNAi lines may serve as powerful genetic tools for epistasis analysis and are important for dissecting defense signal pathways in rice. Here we describe a detailed procedure for generating JA-insensitive transgenic rice lines by RNAi-mediated suppression of a rice ortholog of *Arabidopsis COI1* gene ***(15)*** that encodes a key component of JA signal transduction.

2. Materials

2.1. Construction of RNAi Vector

1. *Escherichia coli* strain DH5α.
2. *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Sal*I restriction enzymes, Taq DNA polymerase and T4 DNA Ligase.
3. 10 m*M* dNTPs.
4. 100-bp and 1-kb DNA ladders.
5. pCAMBIA 1300 vector.
6. QIAquick polymerase chain reaction (PCR) purification kit (Qiagen).
7. QIAprep® Spin miniprep kit (Qiagen).
8. Geneclean III kit (Q-Biogene).
9. Agarose.
10. Luria broth (LB) medium (1.0 L): 10 tryptone, 5 g of yeast extract, 10 g of NaCl, 15 g of agar.
11. Ampicillin.
12. Kanamycin.

2.2. Rice Transformation

1. Seeds of rice (*Oryza sativa* spp. *japonica* cv. Nipponbare).
2. *Agrobacterium tumefaciens* strain EHA105 (rifampicin resistant).
3. Bio-Rad MicroPulser Electroporator and cuvet (gap size 2 mm).
4. YEP medium (1.0 L): 10 g of bacto-peptone, 10 g of yeast extract, 5 g of NaCl, 15 g of agar.
5. Chu (N6) basal salt (C1416), MS basal salt (M5524), and MS modified vitamin powder (M6896; Sigma).
6. Rifampicin: dissolve in methanol to make 25 mg/mL stock solution and store at –20°C.
7. Hygromycin B: 50 mg/mL; store at 4°C.
8. Cefotaxime: dissolve in distilled water to make 250 mg/mL stock solution and store at –20°C.
9. 1 *M* Acetosyringone stock: dissolve 196.2 mg of acetosyringone in 1 mL of dimethyl sulfoxide, store at –20°C.
10. Callus induction medium: 3.98 g of Chu (N6) basal salt, 0.1 g of MS modified vitamin powder, 2 mg of 2,4-D, 0.5 g casamino acids, 2.5 g of proline, 30 g of sucrose, and 2 g of Gelrite. Adjust pH to 5.7, add water to 1 L, and autoclave before use.

11. Suspension medium: 3.98 g of Chu (N6) basal salt, 0.1 g of MS-modified vitamin powder, 0.5 g of casamino acids, 30 g of sucrose, 10 g of glucose, and 100 μ*M* acetosyringone (added after autoclave). Ajust pH to 5.2, add water to 1 L, autoclave before use.
12. Co-cultivation medium: 3.98 g of Chu (N6) basal salt, 0.1 g of MS modified vitamin powder, 1 g of casamino acids, 2 mg of 2,4-D, 30 g sucrose, 10 g of glucose, 100 μ*M* acetosyringone (added after autoclave), and 2 g of Gelrite. Adjust pH to 5.2, add water to 1 L, and autoclave before use.
13. Washing medium: 3.98 g of Chu (N6) basal salt, 0.1 g of MS-modified vitamin powder, 30 g of sucrose, and 250 mg of cefotaxime (added after autoclave). Adjust pH to 5.7, add water to 1 L, and autoclave before use.
14. Selection medium: same as the callus induction medium with addition of 250 mg/L cefotaxime and 50 mg/L hygromycin after autoclave.
15. Regeneration medium: 4.31 g of MS basal salt, 0.1 g of MS modified vitamin, 1 g of casamino acids, 2 mg of kinetin (or 2 mg benzyladenine), 0.1 mg of α-naphthaleneacetic acid, 30 g of sucrose, 30 g of sorbitol, 50 mg of hygromycin (added after autoclave), and 3 g of Gelrite. Adjust pH to 5.7, add water to 1 L, and autoclave before use.
16. Rooting medium: 4.31 g of MS basal salt, 0.1 g of MS-modified vitamin, 30 g of sucrose, 50 mg of hygromycin (added after autoclave), and 2 g of Gelrite. Adjust pH to 5.7, add water to 1 L, and autoclave before use.

2.3. Analysis of Transgenic Rice Plants

1. TRIzol reagent (Invitrogen).
2. 70% and 100% ethanol.
3. TE buffer: 10 m*M* Tris-HCl, 1 m*M* ethylene diamine tetraacetic acid, pH 8.0.
4. 3 *M* Sodium acetate, pH 5.2.
5. PerfectHyb™ plus hybridization buffer (Sigma).
6. 20X standard saline citrate (SSC): dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in dH_2O, adjust pH to 7.0, and add water to 1 L.
7. 10% Sodium dodecyl sulfate.
8. Nylon membrane.
9. Random primed labeling kit.
10. Formaldehyde.
11. JA.
12. Methyl jasmonate (MeJA).

3. Methods

3.1. Generation of Rice COI1 *RNAi Construct*

1. Extract genomic DNA from young leaves of rice seedlings using CTAB method as previously described ***(16)***.
2. Design two pairs of rice *COI1* gene-specific primers containing *Bam*HI/*Kpn*I and *Bam*H/*Sal*I sites, respectively (*see* **Note 1**). To generate intron-containing hairpin RNA, a 1-kb *Bam*HI/*Kpn*I fragment (a fragement, with a 258-bp *COI1* intron)

and a 0.7-kb *Bam*H/*Sal*I fragment (B fragment) were amplified from genomic DNA by PCR under the following program: 94°C for 2 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min; finally 72°C for 10 min (*see* **Note 2**).

3. Check the PCR products on 1% agarose gel for specific amplification and verify by restriction enzyme digestion or DNA sequencing as needed.
4. Purify the PCR fragments (A and B) with QIAquick® purification kit.
5. After being digested with *BamH*I and *Kpn*I, the A fragement (approx 1.0 kb, with intron) is purified with the Geneclean III kit and ligated to the *Bam*HI/*Kpn*I sites of pCAMBIA1300S, which is modified from pCAMBIA1300 and contains a double 35S promoter and a terminator ***(14)***.
6. Transform the ligation product into DH5α-competent cells by heat shock (42°C, 1 min) treatment. Plate the bacteria on LB medium with kanamycin (50 mg/L) and incubate at 37°C overnight.
7. Pick up 10 bacterial colonies for plasmid DNA extraction using QIAprep® Spin miniprep kit and identify the pCAMBIA1300S recombinant containing the *COI1* A fragment by PCR or *Bam*HI/*Kpn*I digestion.
8. Digest the B fragment (approx 0.74 kb) with *Bam*HI and *Sal*I. After purification with the Geneclean III kit, the B fragment was ligated to the *Bam*HI/*Sal*I sites of the aforementioned recombinant plasmid. As a result, the final RNAi construct contains two complementary *COI1* fragments flanking the *COI1* intron, which will allow the formation of inverted repeats or intron-spliced hairpin in rice plants (*see* **Fig. 1A** and **Note 3**).

3.2. Preparation of Agrobacterium *Suspension*

1. Transform the *COI1* RNAi construct into *Agrobacterium tumefaciens* strain EHA105 using the Bio-Rad MicroPulser electroporator according to the manufacturer's instruction (*see* **Note 4**).
2. After electroporation, immediately add 1 mL of YEP or LB liquid medium to the agrobacterial cells and incubate for 2 h at 28°C on a shaker.
3. Plate 50 to 100 μL of agrobacterial suspension onto YEP solid medium containing kanamycin (50 mg/L) and rifampicin (60 mg/L). Incubate the plates at 28°C for 2 d.
4. Pick up several agrobacterial colonies and identify true transformants carrying the *COI1* RNAi construct by PCR and/or restriction digestion. Store the agrobacterial transformant in glycerol stock at –70°C as needed.
5. Streak the agrobacterial transformant on YEP agar medium containing kanamycin (50 mg/L) and rifampicin (60 mg/L) and incubate the plate at 28°C for 2 d.
6. Inoculate one to two loops of agrobacterial cells into 20 mL of YEP liquid medium containing kanamycin (50 mg/L), rifampicin (60 mg/L), and 100 μ*M* of acetosyringone, incubate overnight at 28°C on a shaker (150 rpm).
7. Collect overnight agrobacterial cultures (OD_{600} = 1–2) in sterile centrifuge tubes by centrifugation (<3000*g* for 10 min).
8. Resuspend agrobacterial cells in 30 mL of suspension medium to a density of about OD_{600} = 0.05.

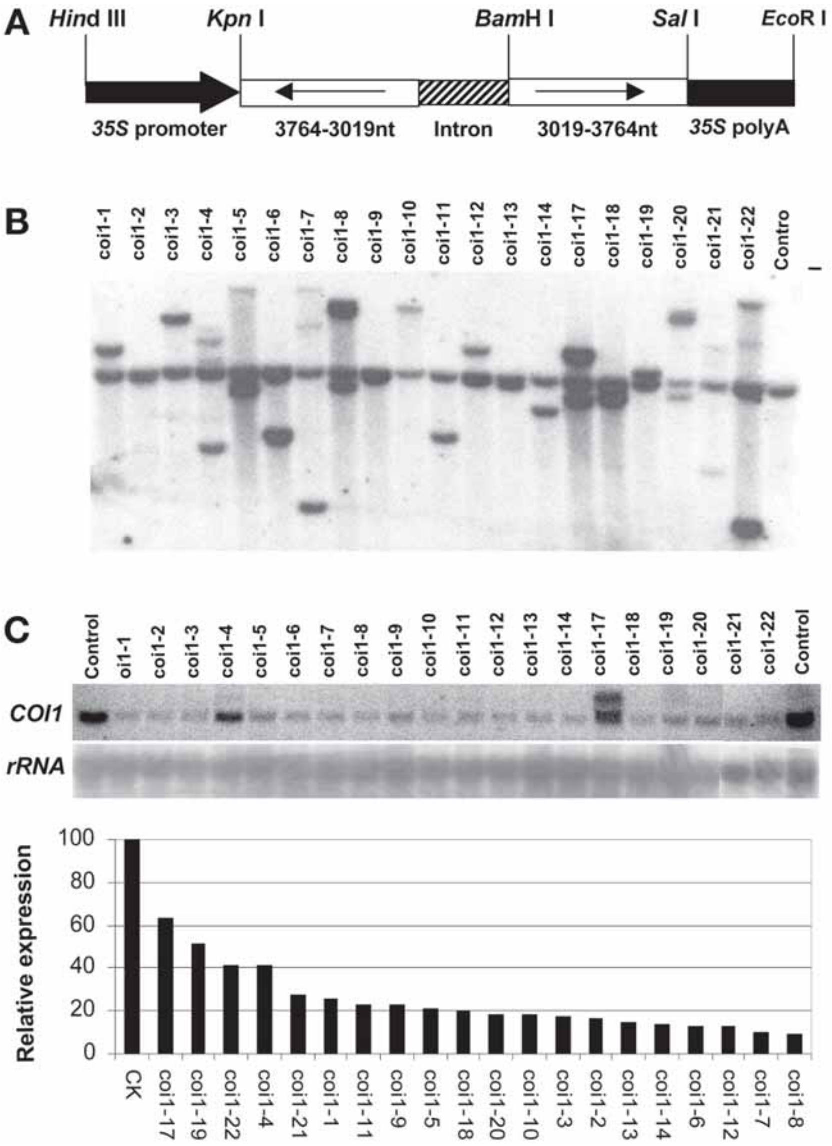

Fig. 1. Suppression of rice *COI1* gene by RNA inference (RNAi). **(A)** Schematic drawing of the *COI1* RNAi construct. **(**B) Southern blot analysis of genomic DNA from the control and *COI1* suppression lines after digestion with *Eco*RI and probed with the B fragment (3019–2764 nt of *COI1*). (**C)** Northern blot analysis of total RNAs from the control and *COI1* suppression lines. A fragment of 825–2244 nt of *COI1* was used as the probe and 25S *rRNA* was used as the loading control.

3.3. *Agrobacterium-Mediated Transformation of RNAi Construct*

1. Dehusk 100 immature or mature seeds of rice and surface sterilize with 70% ethanol for 1 min and then with 50% Clorox® (2.6% sodium hypochlorite) for 30 min with gently shaking.
2. Rinse seeds in sterile distilled water three times to remove residual Clorox.
3. Place seeds on the callus induction medium in 10-cm Petri dishes (10 seeds per plate), seal the plates with parafilm and incubate them under continuous light at 30°C.
4. After 2 wk, separate the calli derived from the scutella with scalpel and transfer them onto fresh callus induction medium and incubate for an additional 2 wk.
5. Select embryogenic calli and soak them in 30 mL of agrobacterial suspension (OD_{600} = 0.05) for 30 min with gentle shaking at room temperature.
6. Decant agrobacterial suspension and blot rice calli on sterile filter papers or Kimwipe tissues to remove excess bacteria.
7. Transfer the inoculated calli onto the cocultivation medium and incubate at 22°C in darkness for 2 d.
8. Collect the cocultivated calli in a 50-mL sterile tube; Wash the calli by gentle swirling for 6 times with sterile 6 × 30 mL dH_2O (1–2 min each time), followed by two-time washes (30 min each) with 2 × 30 mL washing medium.
9. Blot the calli on sterile tissue paper to remove excess washing medium.
10. Transfer the calli onto the selection medium and culture under continuous light at 30°C for 3 wk.
11. Transfer hygromycin-resistant calli to the regeneration medium and culture under continuous light at 30°C.
12. Once shoots are regenerated from calli, transfer them to the rooting medium in test tubes or plastic containers for regeneration of intact rice plantlets.
13. After 2 to 4 wk, rice plantlets are ready for transplanting to soil in pots (*see* **Note 5**).

3.4. *Molecular Characterization of RNAi Transgenic Lines*

1. Perform Southern and Northern blot analyses to verify the introduction of *COI1* RNAi construct into rice transgenic lines and to determine the suppression of endogenous *COI1* gene expression, respectively.
2. Extract genomic DNA from leaves of control and transgenic rice seedlings using the CTAB method ***(16)***.
3. Digest 10 μg of genomic DNA with *Eco*RI in a 30-μL reaction at 37°C overnight.
4. Separate the digested DNA on a 0.8% agarose gel and transfer DNA onto a nylon membrane according to the standard Southern blot protocol ***(17)***.
5. Extract total RNA from leaves of control and transgenic rice seedlings with the TRIzol reagent by following the manufacturer's instruction.
6. Separate 15 μg of total RNA on a 1.2% agarose gel containing formaldehyde and transfer RNA onto a nylon membrane according to the standard Northern blot protocol ***(17)***.

7. Prepare the B fragment used in the *COI1* RNAi construct as a probe for Southern hybridization and the PCR fragment corresponding to 825-1244 nt of rice *COI1* gene as a probe for Northern hybridization (*see* **Note 6**).
8. Radiolabel the aforementioned probes with [α-^{32}P] dCTP using the random priming method ***(17)***.
9. Hybridize Southern and northern blots in PerfectHyb™ plus buffer at 62°C overnight with the radiolabeled probes, respectively.
10. After washing the membranes (2X SSC for 10 min at 62°C twice and then 1X SSC plus 0.5% sodium dodecyl sulfate at 62°C for 20 min twice), the Southern and Northern blots are autoradiographed and/or analyzed with a phosphoimager for relative levels of the *COI1* gene expression in control plant and RNAi transgenic lines (*see* **Fig. 1B,C**).

3.5. JA Sensitivity Test of COI1 Suppression Lines

1. Collect rice seeds from the control plant and RNAi lines with significant suppression of endogenous *COI1* gene.
2. Place surface-sterilized seeds on half-strength MS medium containing 20 µ*M* MeJA (*see* **Note 7**), and incubate at 25°C under the 14-h light /10-h dark condition for 9 d.
3. Measure both shoot and root lengths of control and RNAi transgenic seedlings. In comparison with the control, the *COI1* suppression lines exhibit less inhibition of shoot growth by MeJA and thus are insensitive to jasmonate (*see* **Note 8**).

3.6. Effect of COI1 Suppression on JA-Responsive Gene Expression

1. To determine the role of *COI1* in mediating JA signaling, the expression of JA-responsive genes (e.g., *OsVSP* encoding rice vegetative storage protein, and *OsMPK7* encoding a JA-inducible mitogen-activated protein kinase) are examined in response to JA treatment.
2. Spray the leaves of 2-wk-old control and *COI1* RNAi transgenic seedlings with 0.1 m*M* JA solution.
3. Sample water- and JA-treated young leaves at different time points (0, 1, 3, 6, 12, and 24 h after treatment), freeze them in liquid nitrogen immediately and store at –70°C until use.
4. Extract total RNA from leaf samples and prepare Northern blots as described previously.
5. Hybridize Northern blots with radiolabelled, JA-responsive gene probes (e.g., *OsVSP* and *OsMPK7*).
6. After washing, Northern blots are autoradiographed and analyzed with a phosphoimager for relative expression of JA-responsive genes in control and *COI1* suppression lines following JA treatment. Reduced expression of *OsVSP* and *OsMPK7* are observed in the *COI1* suppression lines in response to JA treatment, suggesting a positive role of the *COI1* in mediating JA-responsive gene expression.

3.7. Disease Resistance Evaluation of RNAi Transgenic Lines

1. Rice RNAi transgenic lines may be evaluated for altered disease resistance and susceptibility using different pathogens, such as *Magnapothe grisea* (rice blast) and *Xanthomonas oryzae* pv. *oryzae* (rice bacterial blight).
2. Preliminary tests can be conducted with first-generation transgenic lines by spot inoculation of *M. grisea* on detached leaves ***(18)***. Further evaluation of disease resistance should be conducted with heterozygous seeds from the first generation transgenic lines and preferably homozygous seeds identified from the second generation transgenic lines (*see* **Note 9**).
3. For the blast infection, 2-wk-old seedlings are spray-inoculated with *M. grisea* at a concentration of 250,000 canidial spores/mL. After incubation in a dew chamber (22°C) for 24 h, rice plants are moved to a growth chamber and maintained at 28°C with a 14-h light/10-h dark cycle.
4. Disease rating as well as measurement of lesion size and number are conducted at 6 d after inoculation. The relative growth of *M. grisea* in control and RNAi transgenic lines can also be determined using a real-time PCR assay or Northern blot/phosphoimaging analysis ***(19)***.

4. Notes

1. Two pairs of specific primers were designed based on the sequence of rice *COI1* gene (accession number BAB84399). The A fragment, corresponding to 2761-3764 nt (with a 258 bp *COI1* intron), was amplified with the first pair of primers (COI1-*Bam*HI-F1, 5'-CCT <u>GGA TCC</u> AGT TAA GTT CCC ACC CAG ATT ATG C; and COI1-*Kpn*I-R, 5'-CCA <u>GGT ACC</u> GGC TAT CCA CAC AGG GTT CTC C). The B fragment, corresponding to 3019-3764 nt, was amplified with the second pair of primers (COI1-*Bam*HI-F2, 5'-CGA <u>GGA TCC</u> GTG AGG AAC GTG ATA GGA GAT AGA GG; and COI1-*Sal*I-R, 5'-CGT <u>GTC GAC</u> GGC TAT CCA CAC AGG GTT CTT CTC C).
2. Gene-specific sequences (e.g., 3' region) are usually selected for specific gene silencing. The inverted repeat should be at least 100-bp long for effective RNAi. Typically, complementary flanking sequences are 250- to 500-bp long and separated by a spacer or intron sequence of 200 to 300 bp. In this case, a 258-bp intron of rice *COI1* gene was conveniently included in the RNAi construct because it was reported to improve the effectiveness of RNAi ***(12)***.
3. Besides the traditional cloning approach, RNAi construct can be made by high throughput cloning using Gateway recombination technology and inverted repeat of a heterologous 3'-untranslated sequence ***(9,10,13)***.
4. Alternatively, a freeze–thawed method can be used to introduce the RNAi construct into *Agrobacterium* cells. Briefly, *Agrobacterium* competent cells are added with 1 μg plasmid DNA and quickly frozen in liquid nitrogen. The microcentrifuge tubes containing agrobacterial cells were then taken out and immediately put in 37°C water bath for 5 min. After addition of 1 mL of YEP liquid medium, incubate the bacterial cells for 2 h at 28°C on a shaker before plating.

5. It is important to keep in moisture after transplanting. Transgenic plantlets should be covered with plastic cones or bags for 2 to 3 d to prevent moisture evaporation and facilitate root growth.
6. To detect the endogenous gene expression without the interference of RNAi transgene, the probe used for Northern hybridization must be different from the gene sequence region used to make the RNAi construct. If the 3' region of a gene is used to make RNAi construct, a DNA sequence from the 5' region should be used as a probe to detect the suppression of endogenous gene expression in Northern analysis.
7. MeJA is much less expensive than JA and is adequate for the jasmonate sensitivity test. Although the growth of rice seedlings can be inhibited by MeJA at as low as 1 μ*M* concentration, 20 μ*M* appears to be an appropriate concentration for examining jasmonate insensitivity in rice.
8. Because the seeds from the primary transgenic plants are heterozygous and contain segregants that lose the RNAi transgene, they need to be further analyzed by PCR for the presence or absence of the RNAi transgene after the JA sensitivity test. Based on PCR results, MeJA sensitivity data can be corrected for the genetic segregation. Therefore, it is better to use homozygous seeds from the second-generation transgenic plants for JA sensitivity tests.
9. To obtain homozygous seeds, rice seeds from the second-generation plants should be harvested individually and tested for homozygosity by PCR. In addition, transgene segregation (the presence or absence of RNAi construct) may be detected based on hygromycin sensitivity. Rice seeds and leaf segments can be placed in Petri dishes containing 50 mg/L hybromycin solution and tested for inhibition of seed germination or browning of leaf segments, respectively.

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16

Virus-Induced Gene Silencing in Plant Roots

Isgouhi Kaloshian

Summary

Virus-induced gene silencing (VIGS) has been used as a quick and easy tool to assess gene function in plants. The virus, carrying a portion of an endogenous gene, triggers a homology-based defense mechanism when introduced into plants. Infection with the modified virus results in gene-specific transcript degradation. Several virus-based vectors have been developed for use in VIGS. One of these virus vectors, tobacco rattle virus (TRV), has a large host range and is transmitted by phytopathogenic nematodes of the genera *Trichodorus* and *Paratrichodorus*. TRV has been used in VIGS to study gene function in above-ground parts of plants. This chapter describes a protocol for the use of TRV vector to silence genes in roots.

Key Words: Tomato; gene silencing in roots; VIGS; virus-induced gene silencing; TRV-based vector.

1. Introduction

Virus-induced gene silencing (VIGS), also known as posttranscriptional gene silencing, is an epigenetic phenomenon that was first described in plants and was referred to as cosuppression ***(1,2)***. Later, it was also discovered to be the cause of cross protection from viral infections ***(3)***. Cross protection, also known as pathogen-derived resistance, has been observed in plants infected with a mild strain of a virus that were found to be immune to subsequent infection by severe strains of the virus ***(4)***. Posttranscriptional gene silencing is a universal mechanism of sequence-specific degradation of endogenous RNA identified in several other organisms ***(5)***. In plants, VIGS of endogenous genes by recombinant viruses carrying portions of plant complementary DNA (cDNAs), quickly emerged as a tool to rapidly reduce message levels of endogenous genes to assess their functional role in a number of plant species ***(6–11)***. The process begins with the introduction of infectious viral particles, carrying a portion of a gene of interest,

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

into a young seedling. As the virus multiplies and the plant grows, the virus spreads to new growing parts and induces VIGS.

Several plant viruses have been engineered as vectors for use in VIGS, including tobacco mosaic virus ***(6)***, potato virus X ***(12)***, tomato golden mosaic virus ***(13)***, tobacco rattle virus (TRV ***[9,14,15]***), barley stripe mosaic virus ***(10)***, cabbage leaf curl virus ***(11)***, and satellite virus-induced silencing system ***(16)***. Among these, TRV infects a large number of plant species and probably has the widest known host range of any plant virus ***(17)***. TRV belongs to the tobravirus group of plant viruses that are characterized by a positive-sense single-stranded RNA and a bipartite genome, RNA1 and RNA2. RNA1 encodes genes required for both replication and movement, whereas RNA2 encodes the coat protein and two nonstructural proteins. TRV can spread in both floral meristematic tissue and root tips, indicating systemic movement throughout plant tissues. This virus is also transmitted by nematodes of the genera *Trichodorus* and *Paratrichodorus,* indicating that TRV is most likely present more uniformly in roots, in particular near root tips, where these nematodes feed. The nematode transmission of TRV and its efficient translocation in roots makes this virus an excellent candidate for use as a VIGS vector to silence genes in roots ***(18)***.

Recently, TRV-VIGS vectors have been improved to efficiently silence endogenous genes. RNA1 and RNA2 cDNAs have been inserted behind duplicated CaMV 35S promoter followed by a ribozyme and nopaline synthase terminator at the C-terminal end ***(9,14)***. Regions of RNA2 coding for nonessential structural genes, including the region associated with nematode transmission, were replaced with a multiple cloning site. These constructs were inserted into binary vectors, pTRV1 and pTRV2, and transformed into *Agrobacterium tumefaciens*. *Agrobacterium* clones containing pTRV1 and pTRV2 are grown separately and mixed at equal concentration in infiltration buffer immediately before inoculation. This and other virus vectors have been used to silence genes encoding metabolic enzymes, such as the phytoene desaturase (*PDS*) gene that results in photobleaching, and genes required for disease resistance ***(19–23)***. Most of the genes targeted by VIGS display phenotypes in aboveground parts of plants.

We have used TRV-based vector to silence the nematode resistance gene *Mi-1* in tomato roots (Kaloshian, unpublished results). *Mi-1* encodes a protein with coiled-coil, nucleotide-binding site, and leucine-rich repeat domains ***(24)***. In addition to conferring resistance to three species of root-knot nematodes (*Meloidogyne* spp.) it also confers resistance to potato aphid, *Macrosiphum euphorbiae*, and whitefly, *Bemisia tabaci* ***(25,26)***. The *Mi-1*/tomato system provided a unique system that allowed us to assay for gene silencing in both leaves and roots of a plant. This chapter describes a method to silence genes in tomato roots using TRV-based vector pTRV1 and pTRV2 (**Fig. 1** ***[9]***).

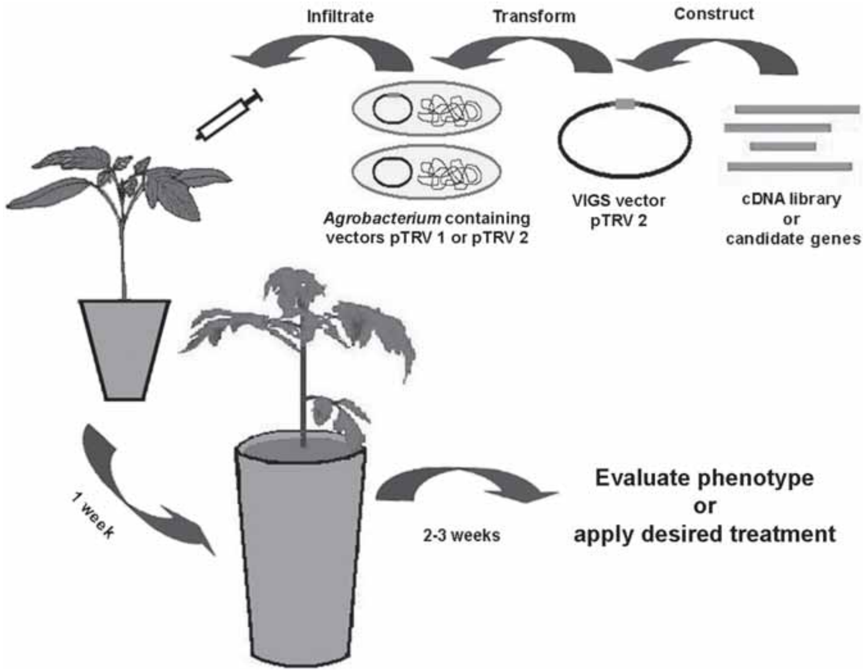

Fig. 1. Schematic diagram of steps involved in tobacco rattle virus (TRV)-based virus-induced gene silencing assays in roots. TRV has a bipartite genome and both are modified and inserted into a binary vector resulting in pTRV1 and pTRV2. A cDNA fragment is cloned into the pTRV2 binary vector and transformed into *Agrobacterium tumefaciens*. pTRV1 is also transformed into *A. tumefaciens*. Bacterial clones containing each vector are grown separately and combined immediately before infiltration into leaflets of 2-wk-old tomato seedlings, grown in seedling flats. The virus moves to the roots and the root tips as it spreads systemically. One week after infiltration, seedlings are transplanted into larger containers. Two weeks after transplanting, plants are ready for root assays.

2. Materials

2.1. Planting and Growth Material

1. Tomato seeds.
2. Organic soil mix.
3. Seedling flats, with 1-in.^{2} well size.
4. Slow-release fertilizer Osmocote® (17-6-10; Sierra Chemical Company, Milpitas, CA).
5. Tomato MiracleGro® (Stern’s MiracleGro Products, Port Washington, NY).
6. Pots.
7. Growth chamber.

2.2. Cloning Into TRV Vector

1. TRV vector: pTRV1 and pTRV2.
2. A 150- to 700-nucleotide DNA fragment of the target gene.
3. Restriction enzyme(s) and buffer.
4. Agarose.
5. 10X TBE (1.0 L): To make 1.0 L of 10X TBE, mix 108 g of Tris base, 55 g boric acid, 40 mL of 0.5 *M* ethylenediaminetetraacetic acid (EDTA), pH 8.0, add double-distilled H_2O to 1 L and adjust pH to 8.3 by adding boric acid.
6. QIAquick Gel Extraction Kit (QIAGEN).
7. T4 ligase and buffer.
8. *Escherichia coli*-competent cells.
9. Luria broth (LB) medium (1.0 L): 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl.
10. Kanamycin.

2.3. Transformation Into Agrobacterium, Growth, and Preparation for Infiltration

1. *A. tumefaciens* strain GV3101 electrocompetent cells.
2. Electroporator.
3. Gentamycin.
4. *Agrobacterium* strain GV3101 containing pTRV1.
5. LB medium.
6. 200 m*M* Acytosyringone (3'-5' dimethoxy 4'-hydroxy acetophenone) in dimethyl formamide.
7. 1 *M* $MgCl_2$.
8. 1 *M* MES (2-[*N*-Morpholino] ethane sulfonic acid).
9. 3-mL Syringe.

3. Methods

3.1. Planting Tomato Seeds

1. Plant tomato seeds individually into seedling flats. Add Osmocote and cover with plastic wrap to avoid drying. Place seedling flat on a tray to catch the drainage water and help maintain adequate moisture. Seedlings with two fully developed leaves are used for VIGS assays (*see* **Note 1**).
2. Fertilize seedlings with Tomato MiracleGro weekly.

3.2. Construction of Virus Vector to Include Target Gene Fragment

1. Choose a 150- to 700-nucleotides region of the target gene (*see* **Notes 2** and **3**).
2. Use appropriate restriction enzyme(s) to cut this fragment to generate either blunt ends or overhangs that could be cloned into the polylinker of pTRV2 vector.
3. Cut the virus vector, pTRV2, with restriction enzyme(s) to generate ends matching the insert (*see* **Note 4**).

4. Run both the insert and the virus vector restriction digests on an agarose gel and cut the fragments and elute DNA fragments using QIAquick Gel Extraction Kit (QIAGEN).
5. Ligate insert and vector for 4 to 12 h.
6. Transform the ligates into kanamycin-sensitive *E. coli*-competent cells, such as DH5α, by either electroporation or heat shock method according to Sambrook et al. ***(27)***.
7. Spread two different aliquots onto LB plates supplemented with antibiotics kanamycin (50 mg/L) and incubate overnight at 37°C.
8. Check the presence of insert in the TRV vector using polymerase chain reaction (PCR) or restriction digestion (*see* **Notes 5** and **6**).
9. Inoculate a positive *E. coli* clone into 5 mL of LB supplemented with kanamycin (50 mg/L) and grow at 37°C for 12 to 16 h with 250 to 300 rpm vigorous shaking.
10. Isolate the recombinant vector as described in Sambrook et al. ***(27)***
11. Transform the recombinant vector into *A. tumefaciens* strain GV3101 competent cells using electroporation.
12. Add LB to 0.5 mL and shake at 250 rpm (28°C) for 2 h.
13. Collect the *Agrobacterium* cells by centrifugation at 11,750*g* in a microcentrifuge for 30 s and discard the supernatant.
14. Resuspend cells in the remaining supernatant and spread on LB plates supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin and incubate at 28°C for 2 to 3 d.
15. Check the *Agrobacterium* transformants by PCR or restriction digestion for the presence of the TRV vector carrying the target gene (*see* **Notes 5** and **6**).

3.3. Introducing Agrobacterium *Into Tomato and Assay for VIGS*

1. Inoculate individually a single colony of each *Agrobacterium* with pTRV1, pTRV2, and pTRV2 containing the target gene, into separate 10-mL test tubes containing 2 mL of LB medium supplemented with kanamycin (50 mg/L) and rifampicin (50 mg/L) and shake the tubes at 250 rpm (28°C) overnight (*see* **Note 7**).
2. Use the 2-mL overnight cultures to inoculate three 125-mL flasks containing 25 mL of LB medium with the same antibiotics as for the overnight cultures, supplemented with 10 m*M* MES and 20 μ*M* acetosyringone, and grow overnight under the same conditions as in **step 1**.
3. Harvest the bacterial cells in sterile disposable 50-mL conical tubes by centrifugation at 2800*g* for 10 min and resuspend in infiltration buffer containing: 10 m*M* $MgCl_2$, 10 m*M* MES, and 200 μ*M* acetosyringone.
4. Adjust the concentration of each culture to OD_{600} of 0.8 to 1.0 and incubate at room temperature for 3 h (*see* **Note 8**).
5. Mix equal volumes (1:1 ratio) of pTRV1 and pTRV2 *Agrobacterium* solutions and use a 3-mL syringe without the needle to infiltrate the solution into the abaxial side of each expanded leaflet.
6. Move the seedlings onto a clean tray and make sure to place each batch of infiltrated seedlings on a separate tray. Maintain seedlings in a growth chamber at 21°C (*see* **Notes 9** and **10**).

7. One week after infiltration, transplant seedlings into desired containers and planting medium and maintain at 21°C (*see* **Note 11**).
8. If further assay is needed to test for the VIGS phenotype, plants are ready for treatment 2 to 3 wk after transplanting. Apply the desired treatment and maintain plants under optimum conditions for the treatment (*see* **Note 12**).
9. At the time of phenotypic evaluation, sample root portion(s) with the expected symptoms for RNA analysis. Sample roots, freeze immediately in liquid nitrogen, and store at –80°C (*see* **Notes 13** and **14**).

4. Notes

1. Use more than one *Solanum lycopersicum* cv. We have observed variation in VIGS efficiency among tomato cultivars (cvs). Best to germinate seeds in a mist chamber. If a mist chamber is not available, seedlings can be germinated in a greenhouse. You can plant a large number of seeds in a single seedling flat. Just before infiltration with *Agrobacterium*, cut sections of the flat with the desired number of seedling to assay with a single VIGS construct.
2. DNA fragments as small as 23 nucleotides have been used to silence the target gene ***(28)***. However, with smaller fragment sizes, silencing is ineffective and larger inserts are recommended for efficient silencing. If the target gene is a member of a gene family, then the 5' untranslated region should be used for silencing.
3. At the time of choosing the DNA region to target in VIGS, determine the region that will be used to assess the transcript level using reverse transcription PCR (RT-PCR). The region targeted for transcript analysis should be different than the one used for silencing, to avoid amplification of transcripts generated by the virus. If targeted sequences are limiting for lack of sequence information or unique sequences, in the case of gene families, overlapping regions could be used. In the latter case, one primer used in PCR should lie outside the targeted area for silencing. With large genes, when using oligo dT for RT, it is advisable to target the 3' region of a gene for PCR amplification to avoid problems with inefficient RT.
4. Currently, there are three TRV vectors available ***(9,14,15)***. The described approach for cloning could be used when targeting a limited number of genes using either one of the TRV vectors. For high-throughput VIGS using cDNA library screens, pTRV2-attR1-attR2 vector with Gateway (Invitrogen) cloning site allows a fast and efficient subcloning alternative ***(9)***.
5. It is highly useful to design primers flanking the pTRV2 polylinker to amplify the insert. If restriction digestion is used to check the presence of insert in the transformed colonies, the miniprep used for this purpose could also be used for the following step.
6. If the original TRV vector components, pTRV1 and pTRV2, are not in *A. tumefeciens* strain GV3101, then you need to isolate the pTRV1 vector and transform into strain GV3101.
7. A culture of *Agrobacterium* with empty pTRV2 vector is used as empty vector control. Although this TRV vector does not cause severe viral symptoms in the tomato cvs we have tested, we have seen differences in the viral symptoms among different cvs.

8. Adjust the concentration of pTRV1 and pTRV2 to the same OD_{600}.
9. Based on establishment of *PDS* silencing in leaves, we identified 21°C to be the best temperature for VIGS in tomato. Although we have not tested the importance of temperature in TRV-VIGS in roots, we assume that efficiency in silencing in leaves also reflects silencing in roots.
10. Catch trays are recommended with TRV-VIGS to avoid contamination because the virus can be transmitted through direct root-to-root contact and root contact with drainage water (*29*).
11. For assays with root-knot nematodes, we use 32-oz plastic cups, with holes in the bottom, and sand.
12. There may be no need to maintain the temperature at 21°C after applying the additional treatment. For example, the outcome of most plant–pathogen or nematode assays is determined within the first few days after application of the inoculum, even if the assays require a period of time before they can be evaluated. Therefore, the result of the assay is based on the efficiency of silencing at the time of application of the inoculum.
13. In general, VIGS in tomato roots and leaves is not uniform. Therefore, it is important to sample roots with silenced phenotype to assess for transcript level.
14. The expression of VIGS in root tissue is maintained for months.

Acknowledgments

This research was supported by a grant from the University of California Agricultural Experiment Station.

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17

Methods for Engineering Resistance to Plant Viruses

Mysore R. Sudarshana, Gourgopal Roy, and Bryce W. Falk

Summary

The development of genetically engineered resistance to plant viruses is a result of efforts to understand the plant–virus interactions involved in "crossprotection," a phenomenon observed with several plant virus diseases. Historically, expression of the coat protein gene of *Tobacco mosaic virus* in transgenic tobacco (*Nicotiana tabacum*) plants is the first example of transgene-mediated resistance to a plant virus. Subsequently, virus-derived sequences of several plant viruses were shown to confer virus resistance in experimental and/or natural hosts. For plant RNA viruses, virus complementary DNA sequences shown to confer resistance include wild-type genes, mutated genes that produced truncated protein products, and nontranslatable sense or antisense transcripts to various regions of the virus genome. Resistance also has been demonstrated for some viruses by mutant trans-dominant gene products, derived from the movement protein and replication-associated protein genes. In addition to virus-derived sequences, gene sequences of plant origin have also been used for transgenic resistance, and such resistance can be virus-specific, for instance, *R* genes isolated from resistant plant genotypes, or nonspecific, for example, ribosome inactivating proteins and proteinase inhibitors. Plantibodies and 2–5A synthetase, a class of proteins of mammalian origin, have also been useful in engineering plant virus resistance. In the case of transgenic resistance mediated by viral coat protein, the mechanism of resistance was suggested to operate during the early events of virus infection. However, transgene-mediated RNA silencing and generation of small interfering RNAs appears to be the primary mechanism that confers resistance to plant viruses. Despite the advantages of transgene-mediated resistance, current interest in the development and use of transgenic virus resistant plants is low in most parts of the world. However, because of its real potential, we believe that this technology will have more widespread and renewed interest in the near future.

Key Words: Coat protein; resistance; antisense; RNA silencing; *R* gene; scFv; proteinase inhibitor.

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

1. Introduction

Plant viruses are intracellular, molecular obligate parasites and cause significant economic losses worldwide. Traditional approaches for managing plant virus diseases include avoiding virus-infected material, chemical control of arthropod vectors and, when available, use of virus-resistance in cultivated crops. However, all of these are labor intensive, and chemical control of insect vectors is becoming more expensive with potential undesirable side effects, including environmental hazards and the generation of insecticide resistance in vector populations and those of other insect pests. The observation of crossprotection ***(1)***, wherein the inoculation of mild virus strains on plants provided protection from more severe strains, suggested that alternative approaches were possible. Several hypotheses were proposed to explain the resistance obtained by crossprotection, but methods to test them were not possible for many years. However, the development of techniques for genetic manipulation of higher plants by using binary vectors derived from *Agrobacterium tumefaciens* allowed the examination of several hypotheses associated with crossprotection. In one such study, expressing the *Tobacco mosaic virus* (TMV) coat protein in *Nicotiana tabacum* plants delayed the onset of symptoms on the transgenic plants when subsequently challenged by TMV ***(2)*** and produced many studies designed to understand crossprotection as well as to develop virus-resistant crop plants of value for agriculture. Subsequently, several different strategies have been attempted for developing virus-resistant plants ***(3,4)*** and this chapter attempts to describe these strategies and their molecular mechanisms.

2. Strategies for Transgenic Resistance

2.1. Coat Protein-Mediated Resistance

It has been two decades since the first report of coat protein (CP)-mediated resistance to TMV in *N. tabacum* ***(2)*** was described, which was immediately followed by reports of CP-mediated resistance for many other plant viruses, most of which have been reviewed by Beachy et al. ***(5)*** and Wilson ***(3)***. In most instances, obtaining resistance seemed to be the primary objective, and studies aimed at understanding the mechanism(s) of resistance are rather scant. At least for TMV, a model virus for understanding plant–virus interactions for the past century, the mechanisms by which transgene expressed CP interferes have been described. The inability of antisense RNA ***(6)*** or nontranslatable + sense RNA sequence of CP ***(7)*** made a strong case for the expression of protein as the factor in imparting resistance. It is widely believed that polysome-mediated virion disassembly is the first step in the establishment of the TMV infection. Studies using TMV RNA and virus particles on protoplasts derived from TMV

CP-expressing plants indicated that resistance was primarily to virus particles, because of a likely interference in the initial events during the infection process ***(8)***. Subsequent studies using in vitro encapsidated TMV genomic RNA containing reporter genes also suggested that virion disassembly was primarily reduced in transgenic plants. However, resistance was limited to the inoculated cell, with no apparent effects on cell-to-cell spread.

Expression of CP appears to be a necessity for successful transgenic resistance to TMV, but reports with other viruses in which nontranslatable CP cistron RNA conferred resistance in transgenic plants ***(9,10)*** suggested that protein expression was not always an absolute requirement for effective resistance. Still, the initial successes with CP-mediated resistance led to the creation of plants expressing different virus CP sequences and resistance to a wide array of RNA viruses. However, CP-mediated resistance does not appear to work for plant-infecting DNA viruses. Still, the success of CP-mediated protection has resulted in the commercialization and squash lines resistant to *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), and *Watermelon mosaic virus* (WMV ***[11]***), and transgenic papaya resistant to *Papaya ringspot virus* ***(12)***.

2.2. Antisense and Sense RNA

Antisense RNA constructs essentially are complementary (c)DNA sequences fused to a promoter so as to be expressed as RNAs complementary to the virus genomic RNA. In addition to various genes, plant virus genomes contain nontranslated region (NTR) sequences required for initiating translation and for genome amplification. Although antisense approaches were not very effective when directed to the TMV CP region, inclusion of the TMV genomic RNA 3'-end NTR proved to be effective ***(6)***. It has to be noted that viral RNA-dependent RNA polymerase binds to the 3'-end NTR of the genomic RNA to initiate the minus-sense RNA synthesis, which is subsequently copied into the genomic RNA. Any delay in the synthesis of RNA would eventually lead to lower virus titer in a given time and hinder the systemic spread of the virus.

In addition to CP genes, several virus nonstructural genes, expressed either as translatable or nontranslatable transcripts, often conferred virus resistance to transgenic plants. One of the best examples is the expression of the TMV 54-kDa gene, corresponding to the carboxyl end of the 183-kDa replicase ***(13)***. Transgenic plants expressing this gene, irrespective of the copy number, showed high levels of resistance to TMV. However, no protein product corresponding to this gene was detectable and the plants were not resistant to a distantly related TMV strain. It was found that in these plants TMV RNA was restricted to the inoculated cells ***(14)***. However, these plants also were found to be exhibiting posttranscriptional gene silencing (PTGS) and the silencing was specific to the antisense strand but not to the + sense strand ***(15)***. Examples of

the sense and antisense RNA-mediated resistance have been reviewed by Wilson ***(3)*** and Baulcombe ***(16)***.

2.3. Interference From Heterologous Viral Genes

Several plant viruses encode specialized proteins known as cell-to-cell movement proteins (MPs). These proteins either interact with secondary plasmodesmata, the intercellular connections between adjacent plant cells, or form tubules to allow intercellular trafficking of virions and/or ribonucleoprotein complexes comprising viral RNA and one or more of virus-encoded proteins. In addition, MPs also bind to RNA and/or DNA. The RNA/DNA binding domain and the plasmodesmata modifying domain being different, the mutants of MPs were thought to bind to plasmodesmata and interfere with wild-type MP-mediated plasmodesmatal trafficking of virus RNA/DNA. A perceivable advantage of this strategy is a broad-spectrum resistance to diverse plant viruses that are dependent on the same type of plasmodesmata for the establishment of infection. Expression of defective TMV 30-kDa MPs, in addition to conferring resistance to TMV, also was able to confer resistance to *Tobacco rattle virus*, *Tobacco ringspot virus* (Family Comoviridae), *Alfalfa mosaic virus* (Family Bromoviridae), *Peanut chlorotic streak virus* (Family Caulimoviridae), and CMV ***(17)***. Among the viruses tested, the members of Comoviridae and Caulimoviridae, instead of using a plasmodesmata-modifying protein, encode a specialized protein that forms tubules allowing trafficking of virions from the infected to adjacent cells. Truncated MPs of plant-infecting DNA viruses of the genus *Begomovirus* (Family Geminiviridae) also have resulted in imparting resistance to homologous as well as heterologous viruses ***(18,19)***. However, it is not known how these plants perform under field conditions. Also, because plants have evolved plasmodesmata as the intercellular communication conduit, interference by MPs may affect plant communication leading to undesirable transgene effects.

2.4. Posttranscriptional Gene Silencing

PTGS was first observed in transgenic *Petunia* plants as a coordinated and reciprocal inactivation of host genes and transgenes encoding homologous RNA ***(20)***. However, PTGS or RNA silencing is a recently recognized strategy for developing virus-resistant plants. Double-stranded RNA generated from a replicating virus, a transgene, or an aberrant RNA can act as a key initiator molecule that is subsequently processed by an RNaseIII-like enzyme to produce 25 nt RNAs known as small antisense RNAs ***(21)***, which were subsequently recognized as small interfering RNAs (siRNAs). The RNA-induced silencing complex, a key component of which is an endonuclease, is then guided by the siRNAs to specifically cleave homologous RNAs ***(22)***.

Involvement of PTGS in virus protection was first evident in transgenic plants using potyviral CP cDNA sequence ***(9,10)***. Lindbo et al. ***(23)*** first proposed PTGS as an antiviral state in plants. This is best achieved when plants are transformed with constructs that express a self-complementary RNA, containing sequences homologous to the target plant virus. Transgene constructs encoding intron-spliced RNA with hairpin structure provided stable silencing to nearly 100% efficiency against homologous plant viruses ***(24)***. Hairpin constructs can be made using generic vectors such as pHANNIBAL and pHELLSGATE ***(25,26)***, and 98 to 853 nt sense/antisense arms in hairpin constructs were efficient in silencing 90 to 100% of independent transgenic plants. In addition to transgene expression, transient expression of double-stranded RNA corresponding to viral sequences, either by mechanical inoculation or by *Agrobacterium*-mediated leaf infiltration, can also impart resistance to plant viruses and has been reviewed recently ***(27)***.

3. Nonviral Genes in Transgenic Resistance

3.1. Genes of Plant Origin That Confer Virus Resistance

The *R* (resistance) genes are dominant plant genes that encode proteins that participate in a general surveillance to identify a specific pathogen. The presence of an incompatible reaction in a specific plant *R* gene–virus combination results in a hypersensitive reaction (HR) where the cells surrounding the initially infected cell are programmed to die, resulting in the isolation of viral infections. Often such responses in resistant plants trigger a systemic acquired resistance that signals a highly resistant state for further virus challenge. The discovery of the resistance gene *N* in *Nicotiana glutinosa* and its subsequent use in the breeding program of cultivated tobacco (*N. tabacum*) virtually eliminated the incidence of TMV. The *N* gene when expressed as a transgene conferred resistance in susceptible genotypes of *N. tabacum*, as well as in tomato (*Lycopersicon esculentum* ***[28,29]***). Similar to the *N* gene, the *Rx* gene encoded product confers resistance to *Potato virus X* (PVX) in potato and in transgenic *Nicotiana benthamiana* and *N. tabacum* ***(30)***. The *Rx* gene product, structurally similar to other *R* gene products, restricts virus in an HR independent fashion. However the gene product has the ability to cause cell death. Despite the association of several *R* genes conferring virus resistance, apart from *N* and *Rx* genes, *Tm-2*^{2} is the only other plant *R* gene that has so far been cloned and shown to confer virus resistance ***(31)***.

In addition to *R* genes, transgenic resistance also has been demonstrated by way of expressing genes that confer resistance to systemic movement of plant viruses, but not involving HR. The gene products of *RTM1* and *RTM2* of *Arabidopsis thaliana*, a model plant for studies on plant development and genetics, resemble the α chain of jacalin, a plant lectin, and a small plant heat

shock protein, respectively ***(32,33)***. These two genes together restrict the long distance movement of *Tobacco etch virus* in *A. thaliana* ***(33)***. However, utility of these genes in transgenic resistance is yet to be shown.

The requirement for *cis* and *trans*-acting virus-encoded proteases in processing viral proteins suggests that protease inhibitors could be used to control virus interactions. Polyprotein processing is a common feature among many viruses but especially for those of families Potyviridae and Comoviridae. A rice cystatin, which inhibits cysteine proteinases, when expressed in tobacco was able to impart resistance to *Potato virus Y* (PVY) and *Tobacco etch virus* ***(34)***. Transgenic plants were not resistant to TMV indicating that the resistance was specific to the two tested viruses of the family Potyviridae.

3.2. Ribosome-Inactivating Proteins

Ribosome-inactivating proteins (RIPs) are RNA *N*-glycosidases that specifically cause removal of the purine base at A_{4324} of 28S rRNA, which results in the separation of the 3'-end of the substrate RNA, thus rendering the rRNA incapable of participating in the translation of mRNA in eukaryotes. Many cultivated crop plants accumulate RIPs in their seeds and various other parts. Pokeweed (*Phytolacca americana*) leaf extract described as antiviral in nature when co-inoculated with TMV, contained a RIP, pokeweed antiviral protein (PAP). The cDNA sequence for PAP was subsequently isolated and cloned and transgenic tobacco and potato plants expressing PAP under the influence of a constitutive promoter showed remarkable resistance to infection by PVX and PVY upon mechanical inoculation ***(35)***. Although these plants also showed resistance to mechanically inoculated CMV, resistance also was noticeable to a varying degree for infection by aphid-transmitted *Potato leaf roll virus* and PVY. In pokeweed, PAP is exported to the cellular matrix and, thus, the pokeweed ribosomes are protected. In transgenic tobacco, upon mechanical injury, transgene-expressed PAP can gain entry into cytoplasm and neutralize the ribosomes before viral RNA can be translated and, thus, resistance is achieved. It is interesting to note that several RIPs of plant origin actually are polynucleotide *N*-glycosylases that can remove adenine bases from DNA and RNA substrates, including those of genomic RNAs of *Artichoke mottled crinkle virus* and TMV and, thus, might affect viral RNAs in the cytoplasm of infected plant cells. A C-terminal deletion mutant of PAP was found to be unable to depurinate host rRNA; however, it was able to inhibit viral infection ***(36)***. It was recently found that PAP also can depurinate capped mRNA in plants and thus possess distinct antiviral properties ***(37)***.

It is also likely that all RIPs are not targeted to the intercellular matrix, or the signal sequence for required for such targeting might be removed to render them cytotoxic. Then virus-regulated expression of a transgenic RIP could

provide virus protection by targeting the infected cells for ribosome inactivation. The AC2 gene product of bipartite geminiviruses (Genus Begomovirus, Family Geminiviridae) is a transactivator of rightward transcription and is required for the efficient expression of the CP, from DNA-A, and BV1, from DNA-B, respectively. The cDNA for a RIP, dianthin, cloned from *Dianthus caryophyllus*, when fused to the promoter of AV1, conferred resistance to *African cassava mosaic virus* in *N. benthamiana* ***(38)***. It is not known whether transactivated RIP-mediated resistance is effective against insect-transmitted virus, the predominant way of geminivirus transmission in nature, nor is it known as to effects on vectors feeding on such plants. Even if some plants were affected, because of the damage caused by the cytotoxicity, such damage could likely be tolerated because of the plasticity of growing plants, which might be a good way to control secondary virus spread in the field. Apart from dianthin and PAP, trichosanthin is another RIP that has been shown to offer protection against CMV and TMV in transgenic tobacco ***(39)***.

Although consumer concerns about the presence of RIPs in food and feed is a strong factor in the development of transgenic crops, it should be noted that RIPs are well distributed among plant kingdom and most grain crops including wheat and barley accumulate RIPs in their seeds.

3.3. Miscellaneous Genes

Apart from *R* genes and RIPs, several other ways have been shown to have potential for transgenic resistance (**Table 1**). This list includes satellites, defective interfering RNAs ***(3,4)***, ScFv antibodies ***(40,41)***, and mouse 2–5A synthetase ***(42)***.

4. Pointers for Strategies for Engineering Virus Resistance

4.1. General Methods for Construction of Plasmid Vectors

4.1.1. Initial Cloning of Genes

It is now certain that it is very much possible to obtain transgene-derived virus resistance using genes and nucleic acid sequences derived from plant viruses, their resistant hosts, and even unrelated organisms. The choice of transgene is dependent on several factors, including (1) the plant host variety or inbred line to be transformed, (2) the type of resistance required, (3) production site of the crop (i.e., greenhouse or open field), (4) the type of commercial commodity obtained from the transgenic crop, and (5) consumer acceptance of the transgenic crop. Resistance obtained by expression of the CP, RNA and self-complementary RNA, is largely dependent on homology of the virus isolates/strains, prevailing in the target area, with respect to the transgene.

It is relatively straightforward to construct desired virus resistance genes if the sequence of the virus is known, and this has been described before in detail

Table 1
Examples of Transgenic Resistance Using Gene Sequences Derived From Sources Other Than Plant Viruses

Gene	Source	Virus	Reference
A. *R* genes of plants			
N gene	*Nicotiana glutinosa*	TMV	28
TM-2[2] gene	*Lycopersicon esculentum*	ToMV	31
Rx gene	*Solanum tuberosum*	PVX	30
B. Enzymes and inhibitors of plant origin			
Pokeweed antiviral protein	*Phytolacca americana*	PVX, CMV, PVY	35
Dianthin	*Dianthus caryophyllus*	ACMV	38
Trichosanthin	*Trichosanthes kirilowii*	CMV, TMV	39
Cysteine proteinase inhibitor	*Oryza sativa*	TEV, PVY	34
C. Mammalian proteins			
2–5A synthetase	*Rattus rattus*	PVX, PVS, PVY	42
scFv	*Mus musculus*	ACMV	40
		TSWV	41

TMV, *Tobacco mosaic virus*; ToMV, *Tomato mosaic virus*; PVX, *Potato virus X*; CMV, *Cucumber mosaic virus*; PVY, *Potato virus Y*; ACMV, *Artichoke mottled crinkle virus*; TEV, *Tobacco etch virus*; PVS, *Potato virus S*; TSWV, *Tomato spotted wilt virus*.

(*43,44*). However, in brief, one can first design two primers for cloning viral genes/sequences of choice using any of the primer design programs. Incorporating restriction sites into the primers to facilitate subsequent cloning is important to consider at this point. Some of the commercially available software that can be used are GCG (Accelrys Inc., San Diego, CA), OLIGO (Molecular Biology Insights Inc. Cascade, CO), Vector NT (Informax Inc., Frederick, MD), and Visual Cloning (Redasoft Corporation, Bradford, Ontario, Canada). If the target virus has an RNA genome, the reverse primer has to be used for reverse transcription followed by polymerase chain reaction (PCR) using a proof reading thermostable DNA polymerase, for e.g., *Pfu* (Stratagene Inc., La Jolla, CA), *Vent* (New England Biolabs Inc., Beverly, MA). Amplification of longer genes (>3 kb) would need a mixture of *Taq* and proofreading thermostable DNA polymerase, for e.g., ElonGase (Invitrogen Inc., Carlsbad, CA), TripleMaster PCR system (Brinkmann Inc., Westbury, NY). The PCR product can be cloned into an intermediate vector for the verification of the sequence and restriction sites.

There are many options for the selection of the binary vector that is going to be used for the transformation, as well as for transcription promoters and

terminators, enhancers of transcription and translation, and markers for selection of transformed plants. Many of the plant virus sequences, gene sequences used in binary vectors, and transformation and regeneration protocols have been patented. It is best to verify intellectual property rights before embarking on generation of transgenic plants. If the purpose is to express protein from the cloned gene, a proper translational context should be provided around the start codon. The sequence of 5'-..AACA ATG..-3' is generally optimal; however, altering the context for some proteins has not necessarily enhanced the protein levels.

4.1.2. Choice of Gene Delivery System

Currently, *A. tumefaciens*-mediated gene delivery and biolistic transformation are the most widely used systems for the delivery of foreign DNA and stable expression in plants. Although these methods are dependent on tissue culture, selection and regeneration of target plant species, electroporation of foreign DNA into meristematic tissue circumvents the need for tissue culture and regeneration. However, only a fraction of the seeds obtained from the primary transformant are transgenic and thus requires careful screening of the progeny.

4.2. Virus Evolution and Engineered Resistance

Most plant virus infections generally occur as the result of entry of several thousand-virus particles into the host. Of these, it is certain that there is nucleotide sequence and biological variation among the infecting population. The occurrence of a heterologous population of virus sequence variants between and within plants (quasi-species nature of some plant viruses) can be a potential problem for transgenic resistance. Of course, this can also be a problem for *R* gene mediated resistance, and therefore must be considered in any resistance-based control strategy. Viruses have evolved to use this variation to take advantage of their environment, some of the population may be able to infect and replicate even under selection pressures imposed by natural and/or transgenic resistance ***(45)***. Thus, understanding virus population dynamics and performing rigorous field trials in crop production sites can help to understand if resistance will be effective, and it must be accepted that even transgenic resistance is not going to be perfect or solve all problems related to controlling plant viruses or other pests/pathogens.

Despite the rapid and significant advancements in developing engineered resistance to plant viruses, only a small number of transgenic virus resistant crop plants have been adopted in agriculture. In the United States, only five transgenic crops, papaya resistant to *Papaya ringspot virus*; potato resistant to *Potato leaf roll virus*; potato resistant to PVY; squash resistant to WMV and ZYMV; and squash resistant to CMV, WMV, and ZYMV, are approved for

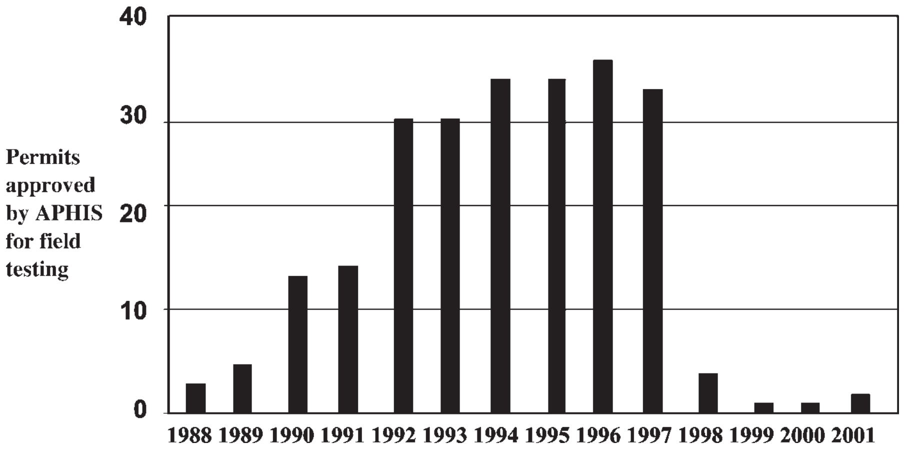

Fig. 1. Bar diagram representing the trend in the number of applications approved by the Animal Plant Health Inspection Service of the United States Department of Agriculture for field-testing of transgenic plants for virus resistance over the years indicated.

complete deregulation for commercial cultivation (http://www.nbiap.vt.edu/cfdocs/). However, during the years 1988 to 2001, 242 applications were granted approval by the Animal and Plant Health Inspection Service Unit of the United States Department of Agriculture for the field testing of transgenic crop plants potentially resistant to plant viruses. Since 1997, the requests for permits for field testing have decreased considerably (**Fig. 1**), which can be attributed in part to the proprietary rights on the promoters, marker genes and sequences of viruses, the lack of interest to identify the operating mechanism of resistance, and/or public perception. However, transgenic resistance is perhaps the best thing to happen as a new approach for controlling plant virus diseases, and in many cases offers more environmentally sound approaches for disease control and in some instances the only possibility. It could go a long way to help keep crop losses stemming from plant viruses much lower than at the present.

Acknowledgments

This work was supported in part by grants from the California Citrus Research Board, and the University of California Discovery Grants Program (BioSTAR).

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18

Development of Genetically Engineered Resistant Papaya for *papaya ringspot virus* in a Timely Manner

A Comprehensive and Successful Approach

Savarni Tripathi, Jon Suzuki, and Dennis Gonsalves

Summary

Papaya orchards throughout most of the world are severely damaged by the destructive disease caused by the *papaya ringspot virus* (PRSV). PRSV-resistant papaya expressing the coat protein gene (*CP*) of PRSV have been used in Hawaii to control PRSV since 1998. This chapter presents the experimental steps involved in the development of transgenic papaya, including transgene construction, transformation, and analysis for virus resistance of the transformed papaya. We also describe the important factors that enabled deregulation, commercialization, and adoption of transgenic papaya to occur in Hawaii in a timely manner. Transfer of this technology to other countries with the similar goal and the development of transgenic papaya in other regions of the world also are described.

Key Words: Biolistic and *Agrobacterium*-mediated transformation; coat protein gene; *papaya ringspot virus*; pathogen-derived resistance; transgenic papaya.

1. Introduction

The papaya industry in Hawaii started in the 1940s ***(1)***, and the *papaya ringspot virus* (PRSV) was discovered in 1945 ***(2)***. By the 1950s, production on Oahu was affected, and the industry subsequently moved to Hawaii island into the area of Puna, which had no commercial production but was free of PRSV. However, by the 1970s PRSV was in the town of Hilo, approx 19 miles away from the papaya-growing area of Puna. Because it was very probable that PRSV would eventually enter the Puna area, research was started by Gonsalves and coworkers in 1978 to develop control strategies for PRSV in Hawaii.

From: *Methods in Molecular Biology, vol. 354: Plant–Pathogen Interactions: Methods and Protocols*
Edited by: P. C. Ronald © Humana Press Inc., Totowa, NJ

Crossprotection was the first approach to be tried, using a mild nitrous acid mutant, designated HA 5–1. The crossprotection strategy using the mild mutant HA 5–1 was used to some extent on Oahu island and in Taiwan ***(3–5)***. However, protection was not sufficient to provide long-lasting economic benefits because of the effort involved in producing mild strains and inoculating plants and the fact that the mild strain produced significant symptoms on some popular cultivars, especially "Sunrise" ***(1)***. This approach was not widely used for longer because of its limitations.

In the mid-1980s, an exciting, but yet unproven alternative approach was used. The report by Powell-Abel ***(6)*** from Roger Beachy's group that transgenic tobacco expressing the coat protein (*CP*) gene of *tobacco mosaic virus* showed significant delay in disease symptoms caused by *tobacco mosaic virus*, spurred us to look towards this approach. The general approach was coined "parasite-derived resistance" (now called pathogen-derived resistance or PDR) by Sanford and Johnston ***(7)***, which offered a new approach for controlling PRSV. PDR is a phenomenon whereby transgenic plants containing genes or sequences of a parasite (in this case, the *CP* gene of PRSV) are protected against detrimental effects of the same or related pathogens ***(8,9)***.

In 1986, Gonsalves and coworkers began using the PDR concept by cloning the *CP* gene of the PRSV mild strain HA 5–1 ***(10)*** and, finally, the resistant transgenic papaya (Rainbow and SunUp) was released for commercial cultivation. PRSV-resistant transgenic papaya in Hawaii became the first commercialized transgenic fruit crop worldwide. The technical details are given later in this chapter. The transgenic work for Hawaii started earlier in anticipation that the PRSV would one day attack the Puna area ***(11)***. In 1992, Hawaii's papaya industry faced a potential economic disaster when PRSV was discovered in the Puna district of Hawaii island, where 95% of the state's papaya was grown ***(11)***. By 1995, PRSV was widespread in Puna, and the industry was in a crisis situation. Fortunately, our research had resulted in the development of a transgenic papaya that was resistant to PRSV at the right time. In fact, an initial field trial of the transgenic papaya was established on Oahu island at about the same time that PRSV was discovered in Puna ***(12,13)***. The sequence of research events (**Table 1 *[11–34]***) and more details for controlling the virus disease and transgenic papaya developments for Hawaii are well documented in reviews written earlier by Gonsalves and coworkers ***(11,14–21)***.

This chapter will mainly focus on the key technical aspects of methodology for genetic engineering the resistance in papaya against PRSV and development of suitable commercial transgenic resistant papaya in a timely manner.

Table 1
Chronology of Key Events in the Production and Commercialization of PRSV-Resistant, Transgenic Papaya in Hawaii

Year	Event	Reference
1940s	The papaya industry was started on Oahu island	22
1945	PRSV disease was discovered	2
1950s	Large production areas on Oahu were eliminated owing to PRSV	11
1950s	Papaya industry moved to the Puna area of Hawaii island	11
mid-1970s	Puna becomes the largest producer (95%) of Hawaii's papaya	11
1978	Work began on control methods for PRSV	11
1983	Mild mutant of PRSV HA (HA 5–1) for cross protection was isolated	5
1985	Concept of pathogen-derived resistance (PDR) codified	7
1985	In vitro translation of PRSV RNA was achieved providing basic information on the coding properties of the viral genome	23
1986	Work toward utilizing the PDR concept was begun by cloning the PRSV HA 5–1 *CP* gene	10
1987	The mild strain PRSV was used for crossprotection to manage PRSV under field conditions	1,3,22
1987	Tissue culture conditions to enable papaya transformation and regeneration was developed	24,25
1990	PRSV-resistant papaya R_0 line 55–1 hemizygous for the *CP* transgene is created by biolistic transformation	26
1991	Transgenic tobacco containing and expressing the functional PRSV *CP* transgene were generated	27
1992	The entire genome sequence of PRSV HA was determined	28
1992	Hawaii papaya industry faced serious disaster due to PRSV in Puna and rouging was begun	11
1992	Greenhouse evaluation of a R_1 line hemizygous for the *CP* transgene of 55–1 was considered	29
1992	First field trial of 55–1 transgenic papaya was conducted in Waimanalo on Oahu island. During this time, cultivars Rainbow and SunUp, hemizygous and homozygous, respectively for the *CP* transgene found in 55–1 were developed.	12,30,31
1994	Hawaii's Department of Agriculture declared PRSV uncontrollable in Pahoa area of Puna and rouging was stopped in this area	11

(continued)

Table 1 (*Continued*)
Chronology of Key Events in the Production and Commercialization of PRSV-Resistant, Transgenic Papaya in Hawaii

Year	Event	Reference
1995	Field trial of SunUp and Rainbow began in Puna on Hawaii island	13
1996	Transgenic line 55–1 and its derivatives were deregulated by APHIS	11,32
1997	Kalapana area, the last area of Puna to be affected was severely infected and rouging was stopped here	11
1997	Exemption from EPA was granted	11
1997	FDA approval was granted for the transgenic lines	11
1998	Bulk seed production of SunUp and Rainbow was completed	33
1998	License agreements were obtained from all parties allowing the commercial cultivation of transgenic papaya and its derivatives in Hawaii only	11
1998	Transgenic seed were distributed free to qualified growers	11
1998	100% of the Puna acreage was nontransgenic Kapoho	20
1999	90% of the farmers obtained the transgenic papaya seed and 76% of them planted them in the field	19
2000	Transgenic papaya made up 50% of the Puna acreage	20
2002	New PRSV-resistant cultivars are developed from the original 55–1 line	34

PRSV, *papaya ringspot virus*; CP, coat protein; APHIS, Animal and Plant Health Inspection Service; EPA, Environmental Protection Agency.

2. Properties of Papaya and PRSV

Papaya (*Carica papaya* L.) is an important fruit crop and is widely grown in countries of the tropics and subtropics ***(35)***. It is a member of the Caricaceae family ***(36,37)*** and believed to be originally from Southern Mexico and Northern Central America ***(38)***. Papaya is a large herbaceous, dicotyledonous plant (up to 3–8 m height), with usually a single erect stem and a crown of alternate large palmate-lobed leaves. The inflorescences are borne in the axils of leaves. The plants are polygamous, with male, female, and hermaphrodite flowers. Wild plants frequently are dioecious, with female or male flowers. Domesticated plants show different sexual types, including hermaphrodite flowers with different masculinity grades, as described by Storey in 1976 ***(38)***. Papaya fruit size ranges from 255 g to 5 to 6 kg, and their color ranges from pale to bright yellow-orange to red.

The papaya fruit is most commonly consumed fresh but it is also processed for making fruit salad, juice, jam, jelly, pie, or ice cream flavoring. Unripe fruits can be eaten raw in salad, cooked in syrup and eaten as a dessert, and the boiled leaves can be used as a vegetable. The fruit has digestive properties because of the presence of papain ***(39)***, and it also has great nutritional value, with high contents of vitamin A, vitamin C, calcium, potassium, and iron ***(40)***.

Papaya trees can easily be cultivated in home gardens from seeds. The tree produces fruit for consumption year round, usually starting at the age of 9 mo. Commercially, when trees are grown at a density of 1500 to 2500 per hectare, annual production can range from 125,000 to 300,000 lbs per hectare. Fruits are harvested for 1 to 2 yr, after which the trees usually are too tall for efficient harvesting. The Food and Agriculture Organization (FAO) estimated that approximately six million metric tons of fruit were harvested in 2002, almost double of the 1980 harvest. Brazil (25.2%), Nigeria (12.6%), India (11.8%), Mexico (11.6%), and Indonesia (8.6%) are the largest producers of papaya ***(41)***. Hawaii is the largest producer of papaya in the United States.

A major limiting factor for papaya cultivation in all geographic areas is a disease ***(42)*** caused by PRSV. PRSV infection is characterized by production of ring spot symptoms that develop on papaya fruits of infected trees ***(2)*** in addition to a range of other symptoms, such as mosaic and chlorosis of the leaf lamina, water-soaked oily streaks on the petiole and upper part of the trunk, a distortion of young leaves that can resemble mite damage, and stunting. PRSV infection may cause lack or severe reduction of fruit production, and fruit that are produced are of poor quality and low sugar concentration.

In nature, PRSV is transmitted nonpersistently by numerous species of aphids to a limited host range of cucurbits and papaya. PRSV also produces local lesions on *Chenopodium quinoa* and *Chenopodium amaranticolor*. The

PRSV virions are nonenveloped, flexuous-rod in shape and measure 760 to 800 × 12 nm. The virus is grouped into two subtypes, PRSV-type P and -type W based on their infectivity. Type P infects cucurbits and papaya, whereas type W infects cucurbits but not papaya ***(42)***. Both P and W type viruses are serologically closely related.

The genome of PRSV consists of single-stranded RNA of 10,326 nucleotides with positive polarity and has the typical array of genes found in potyviruses ***(23,43)***. The genome is monocistronic and is expressed via a large polypeptide that is subsequently cleaved to yield all functional proteins. The 381-kDa polyprotein is processed into eight to nine final products via three virus encoded proteinases (P1, HC-Pro, and NIa). Like other potyviruses, the proposed genetic organization of PRSV RNA is VPg-5' leader-P1 (63K)-HC Pro-P3 (46K)-CI-P5 (6K)-NIa-NIb-CP-3' noncoding region_poly(A) tract (**Fig. 1A *[28]***). There are two possible cleavage sites, 20 amino acids apart, for the N terminus of the CP protein ***(10,23)***. These two sites may be functional; the upstream site for producing a functional NIb protein (the viral replicase) and the other for producing the CP present in aphid-transmissible virions. The complete nucleotide sequence of the PRSV genome has been reported for the following geographical isolates: Mexico (Genebank AY231130), Hawaii (Genebank NC_001785), Thailand strains P (Genebank AY162218) and W (Genebank AY010722), and Taiwan strains P (Genebank X97251) and W (Genebank AY027810). However, CP sequence of numerous strains has been analyzed from various laboratories (**Table 2 *[44–53]***).

3. Papaya Ringspot and Papaya in Hawaii

The PRSV disease was discovered in 1945 ***(2)*** on the island of Oahu, where a papaya industry of approx 500 acres was located ***(11,22)***. By the 1950s, large production areas on Oahu were eliminated, and the industry subsequently moved to Hawaii island into the area of Puna, which had no commercial production earlier. Acreage of papaya increased to 650 by 1960 and to 2250 in 1990. In contrast, the acreage on Oahu fell to less than 50 by 1990 ***(11)***. By the mid-1970s, Puna became the largest producer of Hawaii's papaya, contributing to 95% of the total papaya production of the state.

Remarkably, Puna remained free of PRSV for more than 30 yr. Despite the presence of PRSV in Hilo and Keaau, communities only 19 miles away, Puna remained free of PRSV. The effective physical barrier of the lava rock terrain of Puna, together with the diligence by the Hawaii Department of Agriculture in surveying and rouging infected trees in the Hilo and Keaau areas, kept PRSV from spreading. However, it was very probable that PRSV would someday be found in Puna.

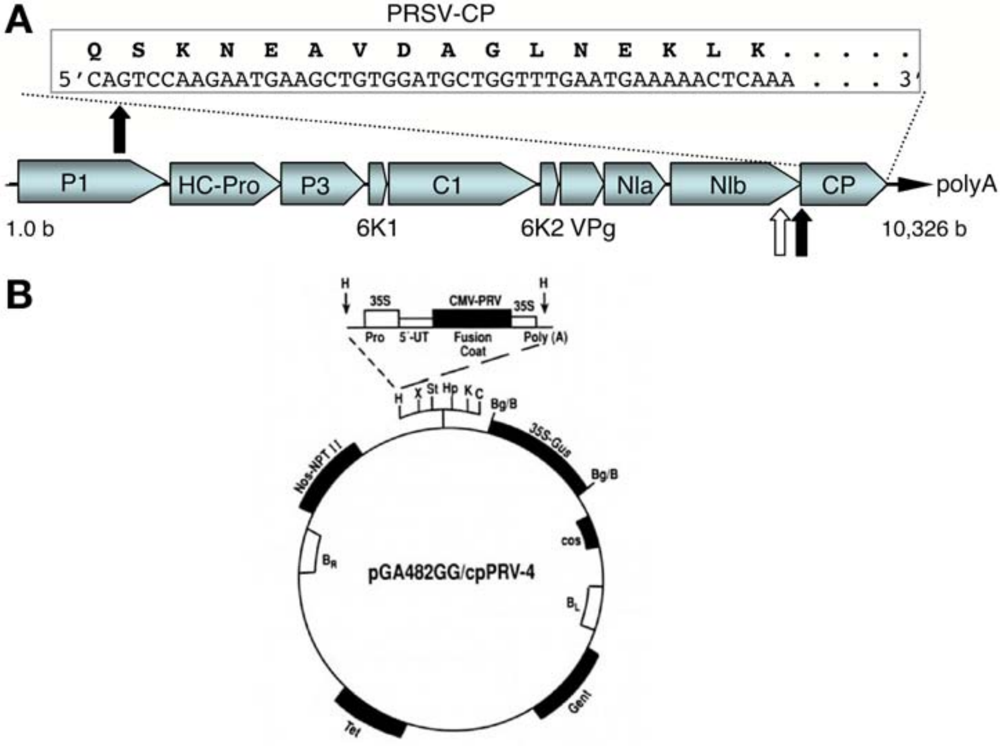

Fig. 1. **(A)** Organization and proteolytic protein products of the 10,326 base monocistonic *papaya ringspot virus* (PRSV) genome. At top shown in detail is the N-terminal sequence of the PRSV HA 5–1 CP. Box arrows represent the proteolytic sites producing the mature coat protein (CP). **(B)** Map of the functional genes of the *Agrobacterium* transformation vector pGA482GG/cpPRV-4 used for generating PRSV-resistant papaya. The PRSV *CP* gene cassette consists of the *CP* structural gene of PRSV HA 5–1 translationally fused to the N-terminal end of the cucumber mosaic virus CP (CMV-PRV) including the translation initiation codon, the CMV 5'-untranslated sequence (5'-UT) and the cauliflower mosaic virus 35S promoter (35S). The CMV-PRV gene cassette is flanked by selectable and visible marker genes, *nptII* and *uidA* (Gus), respectively. B_R and B_L are the left and right borders of the transformation vector T-DNA sequence.

4. Development of Transgenic Papaya for Hawaii

The transgenic work for Hawaii started in 1986 with a team of scientists from different organizations, including Dennis Gonsalves, Richard Manshardt, Maureen Fitch, and Jerry Slightom. Steve Ferreira joined the team in the 1990s.

Table 2
***CP* Gene Nucleic Acid Sequences of PRSV-P Isolates From Various Geographic Regions**

Geographical origin	GenBank accession no.	References
Australia	U14736	44,[b] 45–47
	U14737, U14738	44,[b] 45,46
	U14740	46
Bangladesh	AY423557	47,48
Brazil	AF344640[a]	45,47
	AF344647[a]	45
	AF344647, AF344645, AF344646, AF344641, AF344639, AF344640, AF344650, AF344642, AF344644, AF344643	46
China	AF243496[a]	45,47
	X96538 [a]	49
India	AF305545[a]	45
	AF063220	50,[b] 45–47,49
	AY238880, AY491011, AF238883, AY458617, AY458619, AY238884, AF120270, AY458618, AY238881, AY458620, AY238882, AY238885	47
Indonesia	AF374864[a]	Unpublished
Japan	AB044339 [a]	45–47
Malaysia	AB044342[a]	46
Mexico	AJ012649	49),[b] 45–47
	AJ012650	49,[b] 46
	AJ012099	49,[b] 45,46
	AF309968,[a]	45,46
	AF319468,[a] AF319493,[a] AF319499,[a] AF319502[a]	45
Philippines	AF506902,	45,[b] 47
	AY587583[a]	Unpublished

Sri Lanka	U14741	***44***,[b] ***45***,***46***
Taiwan	AB044341[a]	***45***
	X78557	***51***,[b] ***45***
	X97251	***52***,[b] ***46***,***47***
Thailand	AF506898–900, AF506901, AF506902, AF506862, AF506889,	***45***
	U14743	***44***,[b] ***45–47***
	AB044340[a]	***45***
USA-Florida	AF196839	***53***,[b] ***45***,***46***
USA-HA 5–1	D00595	***10***,[b] ***47***
USA HA	X67673	***51***,[b] ***45***,***46***
USA-Puerto Rico	AF196838	***53***,[b] ***46***
Vietnam	AF506862, AF506889	***45***
	U14742	***44***,[b] ***45–47***

[a]Data not published by submitters.
[b]Reference of submitters.

4.1. Engineering of the PRSV Transgene

The PRSV HA 5–1 strain from Hawaii was used as the source of the *CP* gene for the initial PDR construct because the goal was to create papaya resistant to Hawaiian PRSV strains. PRSV HA 5–1 is a mild virus form derived from the virulent Hawaiian strain PRSV HA by nitrous acid mutagenesis of extracts of infected squash. PRSV HA 5–1 was initially produced for crossprotection studies ***(4)***.

The PRSV HA 5–1 *CP* gene sequence originally was deciphered from a library of complementary DNA derived from purified virus particle RNA and from a partial peptide sequence of a subfragment of the purified CP ***(10)***. Today, *CP* genes from various geographic isolates can be directly amplified from total RNA of infected papaya tissue using polymerase chain reaction (PCR) primers to conserved regions flanking the PRSV *CP* gene thereby bypassing the need for viral particle purification for each new isolate.

The original construct for the PRSV HA5–1 *CP* gene was designed with concept that protein expression was required for PDR, since that was the prevailing thought at the time. Because the PRSV *CP* is produced from the extreme 3'-end of the polyprotein gene by posttranslational protease cleavage, there are no native translation signals specific for the *CP* sequence. Therefore to design a construct for protein expression of the PRSV *CP* transgene, a chimeric gene was made using the translation signals found in the leader sequence (5' untranslated RNA translational enhancer and initial 16 amino acid coding sequences) of the cucumber mosaic virus *CP* gene fused in frame to the structural sequence of the PRSV *CP* including the Q/S protein cleavage site and 51 nucleotides of the noncoding region (nucleotides 9257 to 10168). This was accomplished by cloning the PRSV *CP* structural sequence from plasmid pPRV117 ***(10)*** in between the *Cauliflower mosaic virus* (CaMV) 35S double enhancer promoter–translational leader sequence and CaMV 35S terminator of a cucumber mosaic virus expression cassette ***(54)***. This PRSV *CP* expression cassette was finally cloned into pGA482GG, a modified version of the *Agrobacterium* transformation pGA482 ***(55)*** that contained the *nptII* (neomycin phosphotransferase II) gene behind a nopaline synthase promoter and a *uidA* (β-glucuronidase [GUS]) gene behind a CaMV 35S promoter, used for kanamycin selection and colorimetric screening of transformants, respectively ***(27,56)***. Although we engineered the PRSV *CP* expression cassette (**Fig 1B**) in an *Agrobacterium* transformation vector, we used the plasmid only for biolistic transformation.

Expression of the *CP* from this vector was verified by the use of enzyme-linked immunosorbent assay (ELISA) on the leaves of transformed papaya and tobacco ***(27,57)***. Other groups have shown that different leaders can promote the translation and accumulation of the PRSV *CP* in plants ***(58)***.

Since our original study, breakthrough research by Smith and colleagues ***(59)*** with the potyvirus systems *tobacco etch virus* and *potato virus Y* showed that resistance with potyviruses was mediated by RNA, via the mechanism of posttranscriptional gene silencing (PTGS ***[60,61]***). Indeed, reports on CP-based resistance via protein expression are largely limited to the case of TMV ***(62)***. The authors and others also have reported evidence that PDR in transgenic papaya is mediated by RNA-based mechanisms ***(63–65)***.

4.2. Transformation of Papaya: The Original Approach

An essential element to obtaining the first transgenic papaya was development of tissue culture conditions and identification of a source of papaya tissue that could be efficiently procured, transformed, and regenerated into plants. The main tissue culture parameters were the concentrations of the synthetic auxin, 2,4-dichlorophenoxyacetic acid and sucrose in the induction medium used to proliferate zygotic embryos and somatic embryo cells and the choice of cultivar, which responded differently to these components ***(24)***. The initial study tested embryogenic zygotic embryos, embryogenic calli, and somatic embryos derived from hypocotyls and zygotic embryos ***(24,25)***. In that study, it was established that 2,4-dichlorophenoxyacetic acid-treated zygotic embryos derived from immature seeds of 90- to 120-d-old green fruits had the highest transformation capacity (1.42% of bombarded embryos) after particle bombardment and antibiotic selection compared to embryogenic callus ***(26)***.

The biolistics approach was possible, in part, because the group had ready access to the newly invented gene gun at Cornell University and the help of John Sanford, a co-inventor of the technology ***(66)***. Biolistic transformation involved annealing the purified PRSV *CP* plasmid construct to tungsten particles followed by aseptic bombardment into papaya tissue ***(24,26)***. After bombardment, the tissue was kept on "induction medium" for a total of 4 to 5 wk in the dark without antibiotic selection. The cells were then placed on "maturation medium" with antibiotic selection (75 mg/L kanamycin) for 4 wk in the light and then in maturation medium with higher selection (150 mg/L kanamycin) for 8 wk in the light, during which resistant embryos were able to proliferate. Development of resistant, green plantlets from embryos occurred on a "germination medium" with 150 mg/L kanamycin for 2 to 3 mo. Clones of resistant plantlets were then produced by microprogation and rooted in rooting media.

Leaves of kanamycin-resistant clones were tested for expression of GUS activity, which cause the leaves to turn blue in the presence of the substrate X-Gluc, and by PCR amplification and genomic DNA blot analysis to test for presence of the *nptII* and PRSV *CP* gene. DNA blot analysis also was a useful means to analyze the nature of the integration event (such as arrangment or

copy number). Because integration by the biolistic process is a random event, the relevant PRSV and marker genes of the transformation vector did not always cointegrate; thus, kanamycin resistance was not always correlated with GUS activity or presence of the *CP* gene.

Two transformed lines that were positive for the *CP* gene were further analyzed by RNA blots to determine expression of the transgene. One line showed strong accumulation of the 1.35-kb RNA species predicted for the PRSV *CP* gene transcript.

4.3. Transformation and Tissue Culture of Papaya, Improvements, and Alternate Protocols

Since the original successful transformation and regeneration report, our group reported a modified procedure for the production of somatic embryos that greatly increased transformation efficiency as well as a detailed, updated protocol for biolistic transformation ***(67,68)***. Somatic embryos were produced from zygotic embryos from seeds as in the initial procedure but were bombarded at a step under which active cell proliferation was taking place, 3 d after transfer to fresh induction media. A second difference was that the antibiotic selection period was performed only 7 d after bombardment, but ceased earlier, with the latter induction and maturation steps performed without antibiotic selection. This altered selection scheme allowed for effective amplification of transformed embryos which proliferated vigorously at the later stages in media without antibiotics. Protocols for efficient production of somatic embryos from hypocotyls and subsequent regeneration were developed and found to be efficient for papaya transformation ***(69,70)***. The advantage of using hypocoptyl-derived somatic embryos is that it is easier to procure than the zygotic embryos which have to be individually excised from seeds of immature fruit of the correct age.

It appears that antibiotic selection can reduce the number of transgenic plants that are recovered. Several studies have addressed this issue by limiting the period under antibiotic selection and allowing regeneration of plants to occur without selection as described previously ***(68)***, judicious use of alternative antibiotics as in the case of papaya root explant selection and regeneration ***(71)***, or the elimination of antibiotic selection entirely by use of green fluorescent protein as a visual marker ***(72)***. Selection systems for papaya transformation using alternative antibiotics such as hygromycin have also been successfully tested ***(73)***.

Subsequent to the initial successful transformation of papaya by particle bombardment, protocols for transformation of papaya by *Agrobacterium* were established and improved, providing an alternative means for production of transgenic papaya from hypocotyls, zygotic embryos, or petioles ***(74–76)***. One

potential advantage of *Agrobacterium*-mediated over biolistic-mediated transformation is that, for the most part, the structure of the integrated transgene DNA is predictable, consisting of the marker gene and gene of interest bordered by the *Agrobacterium* binary vector transfer DNA.

A recent study reported high efficiency of cotransformation by the biolistic method of two plasmids into papaya, one carrying a marker gene and the other carrying grape stilbene synthase gene involved in *Phytophthora* resistance (>50%). This new twist to the biolistic transformation approach of papaya might theoretically allow independent insertion of the marker gene and gene of interest and allow segregation and isolation of a marker-free plant ***(73)***.

In addition to improvements in tissue culture techniques and approaches for transformation, application of recent refinements in papaya clonal propagation systems ***(77–79)*** should play a large role in the high-throughput development, screening, maintenance, and distribution of new, pathogen-resistant elite papaya cultivars. For example, efficient micropropagation protocols can allow the propagation of hermaphrodite clones of superior uniformity and elimination of the labor involved in planting and thinning of trees of the undesirable sex ***(34)***.

4.4. Testing of Transgenic Papaya for Viral Resistance in the Greenhouse

In the study leading to the identification of the first pathogen-derived resistant papaya line, all transgenic papaya lines that were positive for the *CP* gene were screened for resistance to the virulent Hawaiian isolate, PRSV HA from which the attenuated strain PRSV HA 5–1 was derived ***(26)***. Six transformants were of the Sunset cultivar and three of the Kapoho cultivar. Four contained the PRSV *CP* gene (two Sunset- and two Kapoho-derived lines), whereas the other 5, along with 35 nontransformed plants, served as controls. Three to fifteen replications were performed on clones of test lines that were produced by micropropagation. Manual inoculation was performed by dusting the four or five youngest fully expanded leaves of test plants with carborundum followed by rubbing with potassium phosphate buffer extracts of *Cucumis metuliferous* leaves infected with PRSV HA. Evaluation of symptoms was performed after 21 d. Of the four transgenic lines tested, one Kapoho line (39–1) was completely susceptible, one Kapoho (19–1) and one Sunset (60–3) line had intermediate (25–33% of the total test plants became infected) levels of resistance, and one Sunset line (55–1) had complete immunity. Of the lines with intermediate resistance, there was a delay of 3 to 17 d before the onset of symptoms. Infection was to some extent affected by the age of the plant at inoculation, with earlier inoculations more likely to cause infection. Line 55–1 was symptomless throughout. Extracts from symptomless leaves of 55–1 as well as 19–1 and 60–3, were tested by a virus recovery assay (in this case, the ability to infect the local lesion host *C. quinoa*) and found to be negative. Although 39–

1 was not resistant, it still showed delayed symptom development compared with nontransgenic controls.

Although line 55–1 was virtually immune to the virulent HA strain of PRSV in the initial study, crossprotection studies have shown that the attenuated HA 5–1 did not effectively inhibit infection by Thailand or Taiwan isolates ***(4,80)***. Thus, line 55–1 was further tested for resistance to other strains of PRSV in a subsequent greenhouse study by Tennant et al. ***(81)***.

To produce the numerous transgenic test plants for this study, the primary transformant (R_0) 55–1 plant, which was female, was crossed with pollen from Sunrise (a sibling line of Sunset). The R_1 transgenic progeny from this cross were identified by the ELISA assay for presence of the *npt*II gene. The transgene segregated at a ratio of 1:1 confirming a single transgene insertion in the original 55–1 plant.

The R_1 progeny hemizygous for the transgene were first rigorously tested by inoculation with the Hawaii PRSV HA isolate because this was to be one of the actual challenges in the papaya fields in Hawaii. Three main approaches to study infectivity of the severe isolate HA were followed:

1. One to two mechanical inoculations.
2. Multiple mechanical inoculations.
3. Inoculation by grafting.

For the first approach, the three youngest fully expanded leaves were inoculated with *C. metuliferous* extracts of different dilutions by the carborundum dusting method. Ten transgenic and 10 nontransgenic plants were tested for each dilution. Symptoms were monitored for 6 wk and plants were reinoculated if no symptoms were apparent. Three weeks after the second inoculation, leaf extracts from these plants were tested for infectious virus by the virus recovery assay. For the multiple inoculation assay, new growth on papaya plants were inoculated every 2 to 4 wk for 10 mo with PRSV infected *C. metuliferus* extracts. For inoculation by grafting, 10 transgenic seedlings were grafted to non-transgenic, HA 5–1 infected trunks. The result of this study showed that there was no replication or movement of the virus in the transgenic plants. The very vigorous resistance of the transgenic papaya to the endemic Hawaiian PRSV isolate added impetus for the initiation of experimental field trials in Hawaii.

The R_1 transgenic papaya also were tested for resistance to other geographical isolates of PRSV. Eleven PRSV isolates were studied: two from Mexico, two from Florida (F, G), one each from the Bahamas, Australia, Brazil, China, Okinawa, Ecuador, Guam, Thailand, Jamaica, and four from Hawaii (HA 5–1, HA, HA-Oahu, and HA-Panaewa). All were serologically indistinguishable from HA 5–1 using antibodies to the HA 5–1. The plants to be inoculated were

5- to 8-wk-old, 6- to 15-cm high, with 6 to 10 leaves. The transgenic R_1 seedlings (10 per virus isolate) were inoculated with the various PRSV strains from diluted extracts of PRSV infected *C. metuliferus* leaves. All plants were observed daily for 6 wk, and symptomless plants reinoculated and tested by the virus recovery assay.

Before the creation of the transgenic papaya, crossprotection was the only other direct means to control the severity of PRSV infection in papaya. Therefore, crossprotection experiments were performed so that resistance by transgenic plants could be compared with a tested approach. Test plants for crossprotection experiments were performed by inoculation of the attenuated HA 5–1 from *C. metuliferous* extracts to nontransgenic papaya. Confirmation of infection was determined by ELISA using antibody against HA 5–1 because plants infected with this virus strain showed little or no overt symptoms. The infected plants were then challenged with *C. metuliferus* extracts of the various geographical isolates exactly as was done for the R_1 transgenic line.

Viruses from Guam, Brazil, Thailand, Ecuador, and Okinawa induced severe symptoms on all transgenic plants, although these were not as severe as that of nontransgenic plants. Australia, China, and Jamaica had an attenuated phenotype on all transgenic test plants but, as in the case of the severe isolates, there was a 7- to 10-d delay compared with the nontransgenic control plants at the monitoring days of 10, 21, and 42 d after inoculation. Complete immunity was found for HA and HA-Panaewa, but severe symptoms were found in 6% of the plants inoculated with the Hawaii strain HA-Oahu. The virus strains from Bahamas, Mexico, and Florida showed a fraction of plants with severe phenotype, whereas others were free of symptoms. Interestingly, the plants without symptoms remained this way after reinoculation. The results of the crossprotection studies were similar to the transgenic studies. However, a major difference was that severe symptoms could be obtained in virus recovery assays from HA or HA-Panaewa symptomless, crossprotected leaves but not from symptomless leaves from transgenic plants. Thus, symptomless leaves of crossprotected plants harbored infective, virulent virus whereas those of transgenic plants did not.

4.5. Initial Field Testing

Although results on the R_1 generation of 55–1 hemizygous (*CP*/+) for the transgene were indicating robust resistance to Hawaii PRSV isolates HA (from Oahu island) and HA-Panaewa (from Hawaii island) field trials in Hawaii were already initiated in attempt to push the progress of the original, promising R_0 transformant 55–1.

Timing was critical because the virus was first identified in the Pahoa area of Puna district on the island of Hawaii, the heart of the state's papaya growing

industry, in May 1992. By October 1994, the state of Hawaii Department of Agriculture declared the Pahoa area uncontrollable and stopped marking trees for rouging, that is, the activity of cutting down infected trees to curb the buildup of virus inoculum.

The purpose of the first field trial initiated in 1992 was to determine whether the resistance first exhibited by the transgenic plants under greenhouse conditions ***(26)*** would hold up under mechanical and aphid transmitted inoculations under field conditions ***(12)***. Although CP-mediated protection in other crops had been evaluated in the field, this field trial was to be a first for a perennial crop, and therefore it was a crucial test for robustness and durability of resistance over a long period. The field trial was conducted at the University of Hawaii experimental station in Waimanalo, situated at 15 m above sea level on the island of Oahu.

The plants for this study were produced clonally by microprogation to insure genetic uniformity. A total of three plant types were tested, including the test plant R_0 line 55–1, which was hemizygous for the transgene, and two controls, a transgenic line carrying no PRSV *CP* gene (Sunset line 62–1), and a nontransgenic parental line Sunset. Inoculation was performed by either of two means, mechanical or vector (aphid transmitted), for each of the three sample types for a total of six experimental treatments. Ten replicates of each treatment set was performed, for a total of 60 plants, arranged in 10 rows, 3 m between rows and 2 m between plants in a row, and randomized with respect to planting and type of inoculation (mechanical vs aphid transmitted). The source of inoculum was from an infected papaya found at the University of Hawaii Horticulture facility. Seedlings were inoculated at approx 4 mo with symptomless plants reinoculated after approx 3 wk. Thereafter, symptoms were monitored every 2 wk during the first 4 mo and at various intervals for 2 yr. Plants were evaluated based on leaf symptoms, the girth of the trunk at 45 cm above ground level and for the presence of PRSV by double antibody sandwich-ELISA. Transgenic plants exhibited normal leaves, had trunk girths averaging 14.6 cm at 18 mo vs 9.3 from sensitive, nontransgenic plants and were negative for PRSV by the double antibody sandwich-ELISA. Manual inoculations resulted in a quick manifestation of infection compared with aphid transmitted infection, but the resulting phenotypes were the same. By the end of 5 mo, all nontransgenic plants were severely infected and near the end of the first year, die back of nontransgenic plants was observed because of weakening and fungal root infections. At the end of 2 yr, in 1994, a complete loss of these plants occurred.

4.6. Development of Cultivars "SunUp and Rainbow"

Given the imposing PRSV presence on the island of Hawaii and the performance of line 55–1 and its R_1 derivatives in field trials and in the greenhouse,

respectively, we made an active attempt to use this new germplasm for the direct development of commercial cultivars during the period of this first field trial. From the greenhouse study on R_1 lines, it was known that the original line 55–1 contained a single copy of the transgene (*cp*/+) in the background of Sunset, a red-fleshed cultivar and showed good resistance to Hawaii isolates of PRSV but not those of other geographic areas. Subsequently, the SunUp variety, which is homozygous (*CP*/*CP*) for the transgene but is otherwise identical to Sunset, was created as the R_3 generation of the original transformant 55–1. This germplasm held the hope for the development of new resistant varieties because crosses with any other nontransgenic variety would yield 100% progeny that would be hemizygous for the *CP* gene (*CP*/+). In Hawaii, the yellow-fleshed Kapoho variety is by far the more popular among farmers and consumers and has a pyriform shape and medium size, which are desirable commercial characteristics for packing and shipping. Thus, in attempt to combine the PRSV resistance and Kapoho characteristics, Rainbow, a F_1 hybrid between SunUp and Kapoho that was yellow-fleshed and hemizygous for the transgene (*cp*/+) was created ***(31)***. The resulting Rainbow cultivar bore pear-shaped fruit with yellow-orange flesh as anticipated and together with SunUp was ready to be tested in field trials to begin in 1995.

4.7. 1995 Field Test in Puna

By late 1994 an application for a field trial was submitted to Animal and Plant Health Inspection Service (APHIS). The field trial was allowed on the stipulations that (1) the test field was sufficiently isolated from nontransgenic commercial orchards to minimize the chance of transgenic pollen escaping to those fields, (2) fruits of abandoned trees left in the area had to be monitored for the possible introgression of the transgene, and (3) all fruits had to be buried on site.

The field trial began with the planting of 3-mo-old seedlings on a portion of an actual farmer's field in Puna in October, 1995 under a permit from APHIS. The test field was at least 0.4 km from surrounding commercial fields. In contrast to the initial trial using line 55–1, this trial involved the transgenic varieties SunUp and Rainbow, and nontransgenic Sunrise, grown by the farmer, and involved mechanical inoculation only at a latter phase, after aphid transmitted virus inoculation was well underway. Other test plants included Sunset, a nontransgenic version of Rainbow (Sunset × Kapoho), and line 63–1, which was a transgenic Sunset containing the *CP* gene of PRSV HA 5–1 and had been obtained during the initial transformation experiments that created line 55–1 ***(82)***. Plants were spaced 1.7 m apart within one row, 8 plants to a row with rows 3.4 m apart. Each two row set was replicated four times in a randomized (complete block) design. This block of transgenic plants was surrounded on all sides by two rows of nontransgenic Sunrise plants (**Fig. 2**).

Fig. 2. Aerial view of the transgenic papaya field trial in Puna, Hawaii. At center is a block of Rainbow plants surrounded by nontransgenic Sunrise, stunted because of *papaya ringspot virus* (PRSV) infection. Adjacent to the field at upper right was a similar block consisting of a nontransgenic version of Rainbow (F_1, Sunset × Kapoho) and a transgenic line similar to Rainbow but with a distinct transgene. The open area in the lower right foreground is the position of the abandoned, PRSV-infected papaya field used as the source virus inoculum source and cleared before flowering of the experimental field.

A similar field situated adjacent to the test plot was set up to simulate a commercial type planting. Altogether, the trial was designed to obtain data relating to how these lines, especially Rainbow, would perform under conditions closely simulating that of commercial production. Like other field tests and unlike greenhouse conditions, many other factors mitigating the health and plant productivity would come into play, such as natural water fluctuations, pest challenges by broad mites and leaf edge roller mites, and root rot caused by *Phytophthora palmivora*. In the field trial, the virus source was a PRSV-infected orchard 24 m upwind of the test plot and the susceptible Sunrise bordering the block of transgenic plants. Mechanical inoculation was not initiated until 2 mo after the initial infection was observed, and then only on every fifth non-infected plant in the Sunrise border rows. PRSV infection first occurred at 3.5 mo after transplanting. PRSV infection symptoms included water-soaked streaking on leaf petioles, chlorotic mosaic and veinclearing on

leaves, leaf distortion and shoestringing of leaves, and ringspots on the fruit. One year after planting, 8 mo after the initial infection, and 3 mo after the initial harvest, all Sunrise plants were infected. None of the SunUp or Rainbow plants were infected during the 2.5-yr period of the trial in the replicated field. However, three Rainbow plants of approx 5000 (in a field not yet culled of female plants) in the block of Rainbow plants tested for commercial production were infected 4 mo after transplanting. PRSV also was observed at a rate of 1.3% on papaya more than 20 mo old; however, symptoms occurred only on some small lateral shoots and not on the main growing shoot. In both types of infection, recovery assays were performed and confirmed the presence of infective PRSV. Fruit data were taken at bimonthly intervals starting at 15 mo after an initial harvest period of 5 mo. Rainbow yields were 126 tons of marketable fruit per hectare per year compared with the average 35.2 tons per hectare per year during the 5-yr period before the discovery of PRSV in Puna (1998–1992). Average fruit weight was 635 to 771 g, and the refractory solids were higher than the 11% minimum of grade A fruit. Nontransgenic Sunrise, on the other hand, was commercially unacceptable 5 mo into the harvest period.

The field trial in Puna was timely as PRSV was ravaging the papaya industry of Hawaii during this period, with the discontinuation of rouging in September 1997 in Kalapana, the last area of Puna district to be infected. In fact, it took only 5 yr from first detection to total devastation of the entire papaya growing area of Puna. Subsequently, a plan was set to move papaya plantings to areas uninfected by PRSV, eradication of papaya and cucurbits in the Puna area, and a 1-yr moratorium on the planting of papaya in the Puna area.

4.8. Greenhouse Analysis of Rainbow and SunUp

The field trial data indicated that the new cultivars SunUp and Rainbow would be viable for direct commercial applications in Hawaii. During this period, a rigorous study also was conducted on the resistance properties of these new lines to other geographic isolates of PRSV under closely monitored greenhouse conditions of the Cornell facilities in Geneva ***(57)***. In this study, resistance of SunUp and Rainbow were evaluated against six PRSV isolates (three from Hawaii, OA, KE, and KA and three from Brazil, Thailand, and Jamaica) by mechanical inoculation (**Table 3**). For the cultivar Rainbow, resistance was only observed against the homologous HA isolate. However, when challenged by the other Hawaiian PRSV isolates, there was a delay in the development of symptoms and a period of 2 to 28 d, during which new leaves did not display symptoms, followed by their reappearance, but in a less severe form.

The symptoms were severe, with no recovery from isolates from outside Hawaii, although a lag in symptom appearance was observed. The resistance exhibited by Rainbow mirrored that of the R_0 line 55–1, both hemizygous for

Table 3
Age-Dependent Resistance Properties of Rainbow, SunUp and Sunrise Papaya to PRSV Isolates From Hawaii and Other Geographic Sources

			Resistance (%) to PRSV isolates						
			Hawaii				Outside Hawaii		
Cultivar	Age (wk)	Height (cm)	HA	OA	KA	KE	JA	BR	TH
Rainbow	6–15	6–9	86	14	0	0	0	0	0
SunUp	3–16	3–20	100	100	100	100	100	100	0
Sunrise	6–15	6–9	0	0	0	0	0	0	0
Rainbow	14	6–20	100	62	33	0			
	17	34–60	100	100	100	100			
SunUp	16	15							0
	23	17–59							75
	29	70–117							100
Sunrise	14–29	24–69	0	0	0	0			0

PRSV isolates HA and OA are from Oahu island; KA and KE are from Hawaii island.
JA, Jamaica; BR, Brazil; TH, Thailand.

the transgene, indicating that the hybrid combination did exert obvious influence on transgene function. In contrast to Rainbow, SunUp was resistant to all PRSV isolates except the Thailand isolate. For the Thailand isolate, there was a long delay of 4 to 6 wk before symptoms manifested. The difference in susceptibility between Rainbow and SunUp suggested that transgene dosage could play a strong role in determining viral resistance. In attempt to understand the basis of the dosage effect, transcription rates of the *CP* transgenes were determined and found to be two times higher in SunUp compared with Rainbow as expected. Paradoxically, transgene transcripts accumulated to a lower steady state level in SunUp as compared with Rainbow. Taken together with the observation that SunUp is more resistant than Rainbow, the evidence suggested that resistance is mediated by a mechanism related to PTGS and that the increased copy number found in SunUp influenced the efficiency of this silencing.

A comparison of the nucleotide sequences of the *CP* genes of the various PRSV isolates tested indicated strongest homology between the HA 5–1 sequence and Hawaii isolates and the least overall homology to the Thailand *CP* gene (89.5%), particularly in the N terminus (83.7%), and a 3'-noncoding region of 35 nt (89.4%). Thus, in this study there was good correlation between the level of *CP* sequence homology and resistance; the closer the homology between the *CP* transgene and the challenging virus, the better the resistance (**Table 4**). A system for producing infectious PRSV RNA transcripts in vitro ***(83)*** allowed for the testing of chimeric contructs consisting of the HA virus genome with all or portions of its *CP* gene replaced with that of an infective isolate, in this case PRSV YK from Taiwan ***(84)***. Like the CP gene from the Thai isolate, YK has a relatively low homology (89.8%) with the *CP* gene of HA ***(52,85)***. The PRSV YK *CP* sequence did in fact confer infectivity to the chimeric HA virus on Rainbow and to a lesser extent SunUp with some segments of the YK *CP* gene appearing to have more influence on infectivity than others. This result indicates that the cognate *CP* sequence of the infecting virus is in fact an important determinant for *CP* transgene-based resistance but that the position of homology might have also play a role. Interestingly, the chimeric virus containing the entire *CP* gene and 3'-untranslated region of PRSV YK caused attenuated symptoms compared to transcripts produced by constructs expressing on YK sequences. These results indicate importantly that viral sequences other than the *CP* gene can also influence infectivity on a transgenic plant expressing the *CP* gene. Recent data strengthens this observation ***(86)***.

Previous results indicated that a R_1 line hemizygous for the *CP* transgene showed better resistance when inoculated at later stages of growth ***(81)***. In this study, Rainbow and SunUp of different ages and sizes (14 wk/13 cm, 17 wk/

Table 4
Summarized Reactions and CP Nucleotide Sequence Homologies of PRSV Isolates to PRSV HA 5–1 Inoculated to Rainbow and SunUp Papaya

	% Homology to transgene *CP*					Reaction to isolates	
PRSV isolates	N	core	C	3'-ncr	overall	Rainbow	SunUp
Hawaii-HA	99.3	99.8	100	100	99.8	R	R
Hawaii-OA	97.3	98.0	100	95.7	97.9	sR	R
Hawaii-KA	95.3	97.1	98.3	93.6	96.7	sR	R
Hawaii-KE	95.3	97.1	98.3	93.6	96.7	sR	R
Jamaica-JA	89.3	95.0	91.5	69.6	92.5	S	R
Brazil-BR	84.4	93.9	98.3	73.3	91.6	S	R
Thailand-TH	83.7	90.7	91.5	89.4	89.5	S	sR

Rainbow and SunUp are hemizygous (*CP*/+) and homozygous (*CP*/*CP*), respectively for the PRSV HA 5–1 *CP* transgene. *N* = 199 nucleotides of the N terminus, core = 641 nucleotides of the core region, *C* = 59 nucleotides of the carboxy terminus, and 3'-ncr = 35 nucleotides of the noncoding regions following the stop codon.

R, resistant; sR, susceptible at young stages and resistant at older stages.

Table was modified from **ref. *57***.

46 cm) and (16 wk/15 cm, 23 wk/38 cm, and 29 wk/93 cm) were inoculated with Hawaii and Thai isolates and evaluated after 56 d. The older and larger Rainbow plants were completely resistant to all Hawaii isolates, whereas the larger SunUp plants were completely resistant to even the Thai isolate. This result concurred with the resistance properties of R_1 plants in the earlier study. The drastic contrast in performance of the Rainbow plants with regards to age graphically demonstrates that subtle factors influence viral resistance. Factors such as these may have contributed to the durable resistance of Rainbow observed under actual field conditions and reemphasizes the importance of the field trials.

5. Deregulation, Commercialization, Impact

In 1995, efforts to deregulate the transgenic papaya were proactively initiated by the research team due to the ongoing devastation of the industry by the killer PRSV and an interest of the papaya industry in getting the transgenic line commercialized. The following subheading is taken largely from parts of Gonsalves ***(11)***.

5.1. Deregulation and Commercialization

Three major governmental agencies namely APHIS, the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA), deregulate the transgenic plants and their products in the United States. APHIS was

largely concerned with the potential risk of transgenic papaya on the environment. Two main risks were of heteroencapsidation of the incoming virus with CP produced by the transgenic papaya and of recombination of the transgene with incoming viruses. A third concern was that escape of the transgenic genes to wild relatives might make the relatives more weedy or even make papaya more weedy because of resistance to PRSV. However, this was of no consequence because there are no papaya relatives in the wild in Hawaii, nor is papaya considered a weed there, even in areas were there is no PRSV. In November 1996, transgenic line 55–1 and their derivatives were deregulated by APHIS ***(32)***.

According to the EPA, the *CP* transgenes are a pesticide because they confer resistance to plant viruses. A pesticide is subjected to tolerance levels in the plant. In the permit application, we petitioned for an exemption from tolerance levels of the CP produced by the transgenic plant. We contended that the pesticide (the *CP* gene) was already present in many fruits consumed by the public because much of the papaya eaten in the tropics is from PRSV-infected plants. In fact, we had earlier used cross protection (the deliberate infection of papaya with a mild strain of PRSV) to control PRSV. Fruit from these trees was sold to consumers. Furthermore, there is no evidence to date that the CP of PRSV or other plant viruses is allergenic or detrimental to human health in any way. Finally, measured amounts of CP in transgenic plants were much lower than those of infected plants. An exemption from tolerance to lines 55–1 and 63–1 was granted in August 1997.

The FDA is concerned with food safety of transgenic products. This agency follows a consultative process whereby the investigators submit an application with data and statements corroborating that the product is not harmful to human health. Several aspects of the transgenic papaya were considered: the concentration range of some important vitamins, including vitamin C; the presence of *uidA* and *nptII* genes; and whether transgenic papaya had abnormally high concentrations of benzyl isothiocyanate. This latter compound has been reported in papaya ***(87)***. Approval by the FDA was granted in September 1997.

In the United States, a transgenic product cannot legally be commercialized unless it is fully deregulated and until licenses are obtained for the use of the intellectual property rights for processes or components that are part of the product or that have been used to develop the product. The processes in question were the gene gun and PDR, in particular, CP-mediated protection. The components were translational enhancement leader sequences and genes (*nptII*, *uidA*, and *CP*). This crucial hurdle involved legal and financial considerations beyond our means and expertise. These tasks were taken up by the industry's papaya administrative committee and its legal counsel, Michael Goldman. The license agreements were obtained from all parties in April 1998, allowing the com-

mercial cultivation of the papaya or its derivatives in Hawaii only. On May 1, 1998, seeds were distributed free to growers who qualified by watching an educational video and signing an agreement that restricted growing of transgenic papaya only in Hawaii. Fruits can be sold outside Hawaii, provided that the importing state or country allows the importation and sale of transgenic papaya.

5.2. Impact of Transgenic Papaya

The development, commercialization, and rapid adoption of transgenic papaya cultivars, that is, Rainbow, SunUp, resistant to PRSV has significant socioeconomic impact on Hawaii. The transgenic papaya in Hawaii was adopted very well by the papaya growers and consumers ***(19,88)***. Adoption by farmers of the transgenic papaya was very high. Adoption was defined as to whether the farmer had planted the seeds, and not based on whether the farmer had signed up and obtained seeds. By September 1999, 90% of the farmers had obtained transgenic seeds and 76% of them had planted (adopted) the seeds. Personal interviews were conducted with 93 of the 171 farmers who had registered to obtain transgenic papaya seeds in 1998. To the question on why they wanted to plant Rainbow, 96% said that it was because of the ability of Rainbow to resist PRSV. A detail about adoption on transgenic papaya in Hawaii can be obtained in an American Phytopathological Society feature story, "Transgenic virus-resistant papaya: the Hawaiian 'Rainbow' was rapidly adopted by farmers and is of major importance in Hawaii today" ***(19)***.

The release of the transgenic papaya resulted in an increase of papaya production in Hawaii and Puna (**Table 5**). In 1992, Puna produced 53 million of the state's 55 million pounds of fresh papaya. The production remained high for 2 yr following the discovery of PRSV in Puna as the result of massive efforts to control the spread of the virus. However, by 1995 papaya production in Puna had decreased to 39 million pounds and was down to 26 million pounds in 1998 when transgenic seeds of cultivars were released to farmers. Production of papaya in Puna increased starting in 2000 and peaked at 40 million pounds in 2001 with 35 million pounds being produced in 2002. The effect of PRSV on papaya production in Puna also can be seen by the decrease in the total percentage of Hawaii's fresh papaya production that was produced in Puna. In 1992, Puna accounted for 95% of the total production, but this figure subsequently dropped to 65% in 1999 followed by a recovery to 84% in 2002.

The impact of the transgenic papaya in increasing papaya production in Puna is also seen by analyzing the relative bearing acres of Rainbow and the nontransgenic Kapoho (**Table 6**). In 1998, Puna production was 26 million pounds from 1640 acres of bearing Kapoho, because Rainbow had not yet produced mature fruit. In 2000, Puna production had increased to 34 million

Table 5
Fresh Papaya Production[a] in the State of Hawaii and in the Puna District From 1992–2002

	Fresh papaya utilization in Hawaii		
Year	Total (×1000 lbs)	Puna (×1000 lbs)	%
(Virus in Puna) 1992	55,800	53,010	95
1993	58,200	55,290	95
1994	56,200	55,525	99
1995	41,900	39,215	94
1996	37,800	34,195	90
1997	35,700	27,810	78
(Transgenic seeds released)			
1998	35,600	26,750	75
1999	39,400	25,610	65
2000	50,250	33,950	68
2001	52,000	40,290	77
2002	42,700	35,880	84

[a]Data were compiled from USDA Statistical Reports of papaya grown in Hawaii (www.nass.usda.gov/hi).

Table 6
Bearing Acres in Puna of Nontransgenic Kapoho and Transgenic Rainbow and the Relationship to Production (1000 lbs) of Fresh Fruit Used[a]

Year	Bearing acres	% Kapoho	% Rainbow	Production
1998	1640	100	0	26,250
2000	1190	32	50	33,950
2001	1675	39	41	40,290
2002	1385	49	37	35,880

[a]Data were compiled from USDA Statistical Reports of papaya grown in Hawaii (www.nass.usda.gov/hi).

pounds from 1190 bearing acres, with Kapoho comprising 32% and Rainbow comprising 50% of the acres. In 2001, 40 million pounds were produced from 1675 bearing acres with Kapoho and Rainbow accounting for 39 and 41% of the acreage, respectively. In 2002, the bearing acreage dropped and the amount of Kapoho rose to 49%, whereas Rainbow remained steady at 37%. Production dropped from 40 million pounds in 2001 to 36 million pounds in 2002. These data suggest that Rainbow accounts for at least half of the fresh fruit produc-

tion in Puna. Furthermore, production of similar amounts of papaya can be obtained with less acreage. This latter observation is attributed to the higher level of production of Rainbow compared to nontransgenic Kapoho.

One of the major contributions that the transgenic papaya has made to the papaya industry is that of helping in the economical production of nontransgenic papaya ***(11)***. This has occurred in several ways. First, the initial large-scale planting of transgenic papaya in established farms along with the elimination of abandoned virus-infected fields drastically reduced the amount of available virus inoculum. The reduction in virus inoculum allowed for strategic planting of nontransgenic papaya in areas that were free of infected plants and were not surrounded by areas of infected plants, such as had been present in 1992 ***(11)***.

Although definitive experiments have not been conducted, it seems that transgenic papaya can provide a buffer zone to protect nontransgenic papaya that are planted within the confines of the buffer. The reasoning is that viruliferous aphids will feed on transgenic plants and thus be purged of virus before traveling to the nontransgenic plantings within the buffer. This approach also has the advantage that it allows the grower to produce transgenic and nontransgenic papaya in relatively close proximity. Timely elimination of infected trees would need to be practiced to delay large-scale infection of the nontransgenic plants ***(17)***.

5.3. Diversified Cultivation of Papaya in Hawaii

The release of PRSV-resistant papaya provided options for papaya growers. Before the release of transgenic papaya, growers on Oahu, for example, farmed only small plots of papaya because of the effect of PRSV on production. Growers on Oahu now enjoy a niche market, growing Rainbow papaya for residents in Honolulu and other urban areas of the island. Besides SunUp and Rainbow, new varieties have since been created starting with Rainbow F_2 plants ***(34)***. Rainbow F_2 female plants were chosen to initiate the backcrossing program since they were 50% homozygous for all characteristics. Multigenerational backcrosses have been made to Kapoho and the variety Kamiya in attempt to transfer the PRSV-resistance to these varieties. Kamiya bears fruit with smooth waxy skin, and deep orange flesh color which is popular on the island of Oahu. Backcrossing with Kapoho was performed in attempt to obtain a PRSV resistant line with firm-fleshed fruit qualities of this parent important for shipping and handling. Other important agronomic traits are that Kapoho is adapted to the shallow, lava fields, whereas Phytophthera-tolerant Kamiya is adapted to deep soils. During the multiyear backcross program, the use of embryo rescue, harvesting seed for tissue culture germination from 2-mo-old, immature fruit rather than waiting for seed from mature, 5-mo-old fruit reduced the breeding

program from 4 to 3 yr. Micropropagation and rooting of cuttings were used to produce clones of random and selected progeny which allowed the testing of genetically identical lines in field trials at different geographical locations. In addition, PCR was used to identify lines segregating for the transgene ***(34)*** as well as to determine the sex ***(89)*** to allow direct targeting of relevant lines. The resulting Kapoho backcross 1 (Rainbow F_2 X Kapoho) and Kamiya F_1 hybrid (Rainbow F_2 X Kamiya) were named Poamoho Gold and Laie Gold, respectively. Micropropagation of these papaya varieties, particularly Laie Gold ensure the production of only hermaphrodite plants demanded by the market, earlier and lower bearing trees with initially higher yields, and provides selected, superior clones that could result in improved quality and yield.

It is important to note that because these PRSV-resistant cultivars derived from the original, deregulated transgene line 55–1, they also could be directly used for commercial application, circumventing the time-consuming and complicated process of approval required if a novel transformation event was involved and allowing the development of cultivars to meet niche market needs on Oahu island and possibly other islands

Since the introduction of transgenic papaya, the number of cultivars available to papaya growers in Hawaii has thus actually increased. As noted earlier, Kapoho accounted for 95% of Hawaii’s papaya market in 1992. Now Rainbow and Kapoho are dominant and the transgenic SunUp, Laie Gold, and the nontransgenic Sunrise make up a small but significant part of the cultivars grown in Hawaii ***(20)***.

6. Important Factors Influencing the Timely Development of Transgenic Papaya in Hawaii

Given the speed and severity of the PRSV epidemic on Hawaii island which began with the discovery of PRSV in Puna in 1992 and reached the level of disaster within a span of 5 yr in 1997, quick action was necessary to bring about the actual commercialization of the transgenic papaya and bring about the repair of the papaya industry.

Key factors contributing to this timely development are outlined below:

1. R_0 plants were directly tested in the field before full characterization. The first report of the transgenic papaya together with initial characterization for resistance was published in 1992 ***(26)***, the year the first field trial on the R_0 line 55–1 was initiated. At this time, it was not fully characterized genetically, and its resistance characteristics were not fully known, particularly to other geographic isolates of PRSV. However, field data were essential in assessing the potential of using transgenic papaya for applied, commercial applications.
2. The first field trial was used to test resistance under field conditions as well as a venue to develop practical cultivars. Two potential cultivars, Rainbow and SunUp, hemizygous and homozygous, respectively, for the PRSV *CP* transgene were

developed while resistance properties were still being performed on the original R_0 plant line. These cultivars showed promising horticultural characteristics and were ready for field testing by 1995. By 1994, data from this first field trial of R_0 plants was near completion with results indicating that line 55–1 showed complete resistance in the field to a PRSV isolate found in Hawaii.

3. Resistance of new transgenic plants to other geographical isolates was rigorously tested at an early stage. Resistance of an R_1 generation of transgenic papaya to various geographical PRSV isolates in addition to those from Hawaii was rigorously tested, concurrent with the initial field trial and development of new transgenic cultivars, and the results were reported in 1994 ***(81)*** at the end of the first field trial. These results reconfirmed the vigorous resistance of the transgenic papaya to local Hawaiian PRSV isolates while providing an early warning on the limitations of their use against other geographic isolates and in other areas. During this period, the R_0 line 55–1 was documented to contain a single transgene.
4. The second field trial in Puna was set up in a timely manner, using actual cultivars of interest and at an actual site of production. The large field trial in Puna was begun in 1995, using the newly developed transgenic-derived cultivars, SunUp and Rainbow only 3 yr after initiation of the first field trial on the R_0 line. Although greenhouse trials on these particular cultivars were ongoing and not completed at this time, the previous R_1 line data ***(81)*** and preliminary data were sufficiently positive to warrant a direct field test under commercial conditions.
5. Deregulation so crucial to commercialization and release was begun well in advance, during the field trial in Puna. Application for deregulation of the transgenic papaya including 55–1 and a second transgenic line derivative 63–1 was begun before the completion of this field trial.
6. Seed production was initiated before papaya was commercialized. Cultivars SunUp and Rainbow were created by the end of 1994 for the beginning of the field trial in Puna. By the end of the final field trial and before the release of the transgenic cultivars in 1998, enough seeds of the cultivar Rainbow to plant 1000 acres were already bulked up on the island of Kauai ***(33)***, ready for distribution to farmers who by this time had been forced to abandon their papaya fields owing to the devastation caused by PRSV.
7. Commitment of researchers and acceptance of the consumers. A major factor in the success of the transgenic papaya in Hawaii was the combination of researchers each which contributed a unique aspect to the project. Personnel at the University of Hawaii monitored the virus epidemic at the forefront and managed field trials (S. Ferreira and K. Pitz) and developed the papaya tissue culture, transformation, and regeneration protocols (M. Fitch and R. Manshardt). Jerry Slightom of the UpJohn company and John Sanford at Cornell University were key members contributing to expertise in biotechnology and such tools as vectors and development of the gene gun and PDR, respectively. Dr. Dennis Gonsalves of Cornell University provided an intimate knowledge and experience in the field of PRSV and PDR. Finally, the transgenic papaya was enthusiastically embraced by both farmers and consumers alike, without whose support and acceptance the success of such an undertaking would not be possible.

7. Development of Transgenic Papaya in Other Geographic Areas

Because PRSV is a worldwide problem on papaya, which is widely grown in the tropics, other countries have showed interest in developing the technology for their use. Thus, a program was set up by one of the authors (D.G.) to develop and transfer the technology to Brazil, Jamaica, Thailand, and Venezuela ***(16)***. This section summarizes the status of the program and the results obtained on transgenic papaya developed in other laboratories.

7.1. Brazil

The *CP* gene of PRSV isolate from the Southeast region of the state of Bahia was used to engineer the PRSV *CP* constructs and transform papaya via biolistic and *A. tumefaciens*-mediated transformation. The resulting R_0 plants appeared to be resistant to the homologous virus as well as to the Hawaiian strain PRSV HA and also an isolate from Thailand ***(90)***. Candidate resistant lines were sent to Brazil in 1999, where they were subsequently analyzed up to the third generation. Thirty-two transgenic papayas were tested in the field in Brasilia in a 900-m^2 plot where they showed good resistance. The main purpose of the initial tests was to produce seeds and then test the plants in producing areas in the states of Ceara, Espirito Santo, and Bahia. Unfortunately, the program has come to a stand still because of the strict regulations imposed on genetically modified organisms (GMOs) by the home regulatory body, the Brazilian Technical Committee for Biosafety (CTNBio). Currently, a small field trial is being carried on at the experimental station at Cruz das Almas. Recently, Brazil has passed legislation to allow further testing of transgenic plants and it is hoped that more intensive work can be resumed to identify and eventually commercialize the resistant line.

7.2. Jamaica

A virus isolate from the island of Cayman was used in the construct. Two versions of the transgene were made: one with a translatable *CP* gene and the other a nontranslatable *CP* gene. Transgenic plants were obtained after bombardment into Sunrise (solo type) papaya somatic embryos. Under greenhouse conditions with manual inoculation, high (78%) resistance was found for the translatable *CP* construct compared with only 10% for the nontranslated construct. However, even for the sensitive plants, a delayed recovery was observed in which an initial sensitive phenotype disappeared in subsequent new growth. R_0 plants were transferred to Jamaica in 1998.

Initial field trial was conducted with R_0 in 1998 and the resistance to field sources of PRSV of the homologous type in the field was similar to mechanical inoculation in the greenhouse with 80% of the transgenic papaya carrying the translatable *CP* gene compared to 44% for the nontranslated construct. Field

trials conducted in 1999 on R_1 plants showed a similar trend to parental lines with 58% resistance ***(91)***. Thus, it appeared that in the case of the transgenic papaya in Jamaica, greater resistance was correlated to translatability of the *CP*. The horticultural, nutritional and other components fell within the range documented for nontransgenic papaya. Results of rat feeding trials showed no adverse health affects attributed solely to transgenic papaya fruit. Taken together, these data indicated that transgenic papaya was safe for consumption.

As in other studies, one goal of the project in Jamaica was the development of local resistant cultivars by crossing in the resistance transgene into locally favored cultivars. Molecular traits deemed useful for such development is the identification of transgenic lines with simple genetics (i.e., a single transgene insert) that can be maintained by selfing and the availability of local cultivars with attributes that are attractive to the local market, which in the case of Jamaica included the large-fruited "Santa Cruz giant" and "Cedro varieties." The target release date of the Jamaica transgenic cultivars for 2002 initially set up in 2000 has been delayed because of the lack of support for a third and final field trial. Thus the release of transgenic papaya in Jamaica is currently on hold.

A further complication is that Europe is a significant market for Jamaican papaya, and unless the papaya is deregulated by the European Union, transgenic papaya will not be able to be exported to Europe or elsewhere. At this moment, there is still a "GMO controversy" with the deregulation of transgenic products in Europe. Conceivably, this could hold back the commercialization of transgenic papaya in Jamaica for fear that the transgenic papaya from Jamaica may somehow be mistakenly shipped to Europe before it is deregulated in Europe.

7.3. Thailand

Two popular cultivars, "Khakdum" and "Khaknun," were targeted for transformation using the nontranslatable *CP* gene of a PRSV isolate from Northeast Thailand. Several transgenic resistant lines of *CP* transgenic Khakdum and Khaknun were identified in greenhouse inoculation tests at Cornell University, New York. A number of potential R_0 lines were delivered to Thailand in July 1997. Work was immediately started by Dr. Nonglak and Ms. Prasartsee on the propagation, seed increase, and testing for resistance of the potential lines. Ms. Prasartsee has headed the work since 1997. By 1999, field trials of the R_1 generation showed excellent results. By the year 2002, an R_3 line of Khaknun had been selected and showed excellent PRSV resistance and horticultural characteristics. In comparative tests, the transgenic line showed that 97% of the progeny were resistant under intense disease pressure and yielded 63 kg of fruit per tree in the first year, whereas nontransgenic papaya yielded only 0.7 kg per year. Crosses between independent lines of Khakdum recently have been

shown to also show good resistance under greenhouse and field conditions (V. Prasartsee, unpublished results).

Concurrently, molecular characterization, biosafety experiments, analysis of transgenic fruit for food properties and food safety, and intellectual property rights were initiated using material that has been selected for eventual deregulation and commercialization. Nearly all biosafety experiments that are mandated by the national committee on biosafety have been completed. Tests on food safety and other characteristics, such as vitamin, amino acids, soluble solids and other profiles, are being done and should be completed in the near future. However, recent controversial events relating to GMOs will very likely slow down the process of deregulating the transgenic papaya.

7.4. Venezuela

The *CP* gene of the two isolates were cloned in the plant transformation vector in sense/translatable, sense/untranslatable and antisense forms ***(92)***. The plant transformation vectors with the cloned genes were sent to Venezuela where they were employed to transform a local papaya "Thailandia Roja" via *A. tumefaciens*. A few putative transgenic lines were recovered in 1997 and subsequently crossed. The R_0, R_1, and R_2 generations were molecularly characterized and tested for their resistance against PRSV strains from Venezuela, Hawaii, and Thailand ***(93)***. The resistance seemed to be RNA mediated, and R_1 and R_2 plants showed a promising level of resistance not only to local isolates but also to different geographic isolates of PRSV such as isolates from Thailand and Hawaii ***(93)***. Two hermaphrodite plants showing high level of resistance from the R_2 generation were identified and kept for further multiplication and testing. A small-field plot test was conducted at Lagunillas, Merida with a special permit from the Ministry of Health of Venezuela. The plot was planted with R_1 individuals previously selected in the greenhouse as PRSV-resistant. Later on, the performance of these plants was very good in the field under the local virus pressure. When the transgenic PRSV-resistant papaya were set to flower, unexpected problems started to emerge by the GMO activists and the field trial was set on fire by some of their members in December 2000 ***(93)***. Currently, Dr. Fermin has seeds of resistant plants from R_2 generations, but their use in field trials is still in doubt.

7.5. Taiwan

Transgenic papaya resistant to PRSV were successfully developed by Dr. Shyi-Dong Yeh's team of the National Chung Hsing University following an approach similar to that used for developing the cultivars Rainbow and Sunup for Hawaii, i.e., by use of CP-mediated resistance. In his lab the *CP* gene of a local PRSV isolate, YK was inserted as a transgene in the Taiwanese papaya

cultivar, Tainung No. 2. Transgenic Tainung No. 2 papaya were obtained by *Agrobacterium*-mediated transformation rather than the biolistic method used for developing Rainbow and SunUp. Transgenic, resistant papaya lines carrying the *CP* gene of PRSV YK were generated and four transgenic lines resistant in greenhouse experiments were evaluated under field conditions for their resistance properties and fruit production from 1996 to 1999. Performance of the transgenic lines in the field trials was found to be similar to that of Rainbow and SunUp. None of the transgenic lines showed severe symptoms of PRSV infection, whereas 100% of the nontransgenic plants were severely infected 3 to 5 mo after planting. The transgenic plants (20–30%) did exhibit mild symptoms in the first and second field trials but this did not reduce the yield or fruit quality of these plants. The transgenic lines were not only protected from virus infection, but also produced 11–56% more marketable quality papaya compared with nontransgenic papaya ***(94)***.

In another transformation experiment, Dr. Yeh's laboratory obtained 45 putative transgenic lines, which exhibited PRSV-resistance ranging from delay of symptom development to complete immunity after inoculation in the greenhouse. Similar to Rainbow and Sunup, molecular analysis of nine selected lines that exhibited different levels of resistance revealed that the expression level of the transgene was negatively correlated with the degree of resistance, suggesting that the resistance is manifested by a RNA-mediated mechanism. Segregation analysis showed that the transgene in immune line 18–0–9 had an inheritance of two dominant loci and the other four highly resistant lines had single dominant loci. Seven selected lines were tested further for resistance to three heterologous PRSV strains that originated in Hawaii, Thailand, and Mexico. Six of the seven lines showed varying degrees of resistance to the heterologous strains, whereas one line, 19–0–1, was immune not only to the homologous YK strain but also to the three heterologous strains ***(64)***. In contrast to the transgenic papaya Rainbow, line 19–0–1 from Taiwan was reported to be immune to other heterologous isolates of papaya, whereas Rainbow and Sunup showed a very high degree of durable resistance to the homologous isolates of PRSV as other lines. So far none of the transgenic plants from Taiwan have been deregulated or commercialized.

7.6. Australia

PRSV was only described in Australia as late as 1991 and as yet has not been a major factor limiting production of papaya there. Unlike the spread of virus typical in other countries, strict quarantine measures in Southeast Queensland, where PRSV was first discovered has restricted its spread to the major production region of tropical North Queensland. Nevertheless, given its history in other parts of the world, researchers in Australia have been active in the production

of transgenic papaya that are resistant to Australian PRSV isolates. To this end, transformation and regeneration techniques for papaya were developed. Based on *CP* nucleotide sequence data comparisons of isolates from within and outside of Australia, domestic PRSV isolates have been shown to vary by only 2%. The PRSV isolate used for transgenic studies was obtained from Southeast Queensland. The transgene was designed with a premature stop codon in the PRSV *CP* sequence ***(63)***, thus it was expected that a functional CP protein would not be expressed. Transformation was facilitated by biolistic transformation of secondary somatic embryos of cultivars GD3–1–19 and ER6–4, local cultivars that are also used for production and breeding.

Only two resistant lines were regenerated for each cultivar, each with multiple inserts, and interestingly each male plant. According to RNA blot analysis, the plants with the best resistance exhibited the least detectible message, strongly suggestive of the involvement of an RNA silencing mechanism for viral resistance. Copy number appeared also to play a role in the level of resistance of R_0 plants, as those with single copies were more susceptible. This observation of gene copy number dependence is consistent with that found for RNA-mediated silencing and PRSV resistance of the original Hawaiian transgenic papayas ***(63)***.

7.7. Florida (United States)

Surveys of the PRSV *CP* gene sequences of Florida isolates indicate a closer relationship to PRSV sequences from Puerto Rico and Mexico compared to those isolated from more distant locations. Thus, this study on transgenic papaya carrying the PRSV CP of a Florida isolate was done with the intention of producing of cultivars resistant to PRSV of the Caribbean region. The source of the CP sequence was the Florida PRSV H1K isolate. Four different types of constructs, a sense, antisense, frame shift and stop codon mutation of the *CP* gene were made. The construct was transformed into immature zygotic embryos of the experimental cultivar F65, which is an ancestor of the PRSV-tolerant cultivar Red Lady, by *Agrobacterium*-mediated transformation ***(65)***. None of the resulting plants were immune when inoculated with the homologous PRSV at 10 wk, but moderate-to-highly resistant individuals were identified from among each construct type when inoculation was done at later stages. Of interest, the lines derived from sense and antisense constructs were found to be infertile. The remaining stop codon and frame shift mutation constructs lines were fertilized with pollen from the local cultivars Red Lady and Experimental No. 15 and cultivars grown at the University of Puerto Rico including “Puerto Rico 6–65,” “Tainung No. 5,” “Solo 40,” and Sunrise. The Puerto Rico 6–65 and Tainung No.5 are PRSV-tolerant varieties, whereas Solo 40 and Sunrise are highly sensitive. Resistance of the R_1 progeny seemed to be influenced to

some extent by the particular cross, with progeny from the tolerant Tainung No. 5 and sensitive Sunrise more sensitive than that of other combinations.

7.8. Hong Kong (China)

Researchers in China reported the first case of using the PRSV replicase gene for the production of PRSV-resistant papaya. The replicase gene was cloned from pumpkin leaves infected with PRSV ***(95)***. For the papaya replicase construct, the 3'-end of the gene was deleted and additional codons were added to the 5'-end of the gene. Transformants were obtained by *Agrobacterium*-mediated transformation of embryogenic calli of the cultivar Tai-nong-2. The resulting transformants showed varying levels of resistance in response to mechanical inoculation, including apparent complete immunity in the greenhouse ***(95)***. The mechanism of resistance, whether protein or RNA-mediated, is unknown. The greenhouse test seems to be encouraging, but the fate of these transgenic lines under natural field conditions will have to await results of field data.

8. New Approaches

PDR has been successful for controlling plant virus diseases by exploiting different genes coding for viral proteins, but the *CP* gene is, by far, the most widely used to engineer transgenic resistance ***(96)***. The transgenic papaya in Hawaii is a successful example for the utilization of PDR in controlling PRSV. In that case, the CP was expressed in transgenic plants. It is now conclusive that transgenic resistance in papaya is RNA mediated through posttranscriptional gene silencing. In fact, the prevailing mechanism for transgenic resistance is via posttranscriptional gene silencing or PTGS ***(61,62,97)***. As shown by our work in Hawaii, Rainbow, which is hemizygous for the *CP* gene, had a much more narrow base of resistance than SunUp, which is homozygous for the same *CP* gene. Thus, our early approaches for the developing countries have been to use the *CP* gene from PRSV strains of the target countries. In our quest to develop transgenic papaya with a broader range of resistance, we took advantage of our work on tospoviruses, which is described in the next section.

8.1. Segmented Gene Approach

Evidence that viral transgenes could only confer resistance against closely related viruses stimulated new approaches to create transgenic plants that could be simultaneously resistant to different viruses. The first approach involved the use of multiple *CP* or nucleoprotein (*N*) transgenes regulated by independent promoters and terminators ***(98–100)***. Two main disadvantages precluded the extensive use of this approach: concerns about the introduction of increasingly higher amounts of foreign DNA into crop plants, and limitation on the number

of *CP* genes that could be simultaneously transformed into the plant. A second, more feasible approach toward multiple virus resistance was developed when it was realized that virtually any segment of DNA derived from the *N* gene of *tomato spotted wilt virus* (TSWV) conferred resistance to TSWV in transgenic tobacco ***(101)***. These *N* gene fragments could be as short as 100 bp if they were fused to a longer fragment of non-related DNA, such as the 720 bp *GFP* gene ***(102)***. As a consequence of this finding, chimeric transgenes consisting of the CP genes of *turnip mosaic virus* fused to a 217-bp segment of the *N* gene of TSWV were designed and shown to confer resistance to both viruses in *Nicotiana benthamiana* ***(103)***. The strategy has been extended to make transgenic *N. benthamiana* plants resistant to three different tospoviruses ***(104)***.

Experiments are underway to test the strategy of pasting multiple 240-bp long fragments of *CP* genes from different geographical isolates in different combinations and orientations for their efficacy in PRSV resistance (D. Gonsalves, unpublished results). Resistance is predicted to be specific for all those isolates that share a high degree of similarity to any of the fragments in the chimeric gene. The downside of this approach is that even though segments of the chimeric gene are short, there is still a limitation on the number of PRSV segments which can be engineered together and consequently there is still a limitation to which PRSV isolates a given transgene construct with be effective against. Even so, this is the first rational approach to widen transgenic resistance without increasing the amount of foreign DNA delivered to the target plant. Papaya plants transformed with multiple PRSV *CP* genes fragments are still under evaluation, but the first virus challenges indicate that we have been able to widen resistance to PRSV using the transgenic system described here.

8.2. Synthetic Gene Approach

A new approach to widen the range of resistance involving the creation of transgenes with a single, short DNA segment (~250 bp) able to confer resistance to multiple viruses is currently being tested. This approach does not involve the use of native *CP* genes, chimeras or multiple transformations with selected sequences and would reduce the amount of foreign DNA inserted in the plant. Instead of searching for a natural variant of the *CP* gene able to confer a broad-spectrum resistance, we created it by rational design ***(105,106)***

To test the feasibility of this approach, we used *N. benthamiana*, and the transgenes encoding the third fourth of the TSWV *N* gene which has been previously shown to confer resistance to TSWV ***(101)*** as a model system ***(105,107)***. On the basis of the nucleotide sequence of the *N* gene fragment, we designed and synthesized a novel sequence from oligo nucleotides that was highly similar (90% in this case) to the corresponding fragments of three distantly related tospoviruses, TSWV, *groundnut ringspot virus*, and *tomato chlorotic spot*

virus, which are normally only approx 78% similar at the nucleotide level. The aim was to introduce nucleotide changes at specific locations that would create a synthetic gene equally distant to all sequences used for its creation. Importantly, the nucleotide changes were designed to create short stretches of 20 nt or more of total identity to the different genes at different points along the synthetic gene, which is a prerequisite for targeting RNA degradation by PTGS. This synthetic sequence allowed us to obtain transgenic *N. benthamiana* plants resistant to two different tospoviruses *groundnut ringspot virus* (24% resistance) and TSWV, and potentially to a third one (TCSV) that could not be used in our laboratory.

We have designed a PRSV-derived single sequence aimed at targeting different isolates of the virus via PTGS ***(106)***. The engineered sequence is equally distant at the nucleotide level to all PRSV isolate sequences that we compiled and used for its design. We targeted the variable (5') and conserved regions (3') of the PRSV *CP*. The two synthetic genes were each independently cloned in tandems of three, and transcriptionally fused to a silencer gene (D. Gonsalves, unpublished results) which in this case, was half the *N* gene of TSWV ***(102)***. The two different constructs have already been cloned in a plant expression cassette and somatic papaya embryos have been transformed by the biolistic method. Results derived from these experiments might lead to the design and creation of a synthetic gene in which we would be able to play not only with sequence similarity, but also with the secondary and tertiary structures of the protecting transgene. The long-term goal of these experiments is to create a short, synthetic sequence able to confer universal and durable resistance to PRSV.

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